PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 5/10, 7/01, 15/00
C12N 7/04, 15/33, 15/85

A1

(11) International Publication Number: WO 90/12087

(43) International Publication Date: 18 October 1990 (18.10.90)

(21) International Application Number: PCT/US90/01867

(22) International Filing Date: 5 April 1990 (05.04.90)

(30) Priority data:
334,006 5 April 1989 (05.04.89) US

(71) Applicant: NOVACELL CORPORATION [US/US]; 2341 Stanwell Drive, Concord, CA 94520 (US).

(72) Inventor: HENDERSON, Daniel, R. ; 1196 Grove Circle, Benicia, CA 94510 (US).

(74) Agents: RAE-VENTER, Barbara et al.; Cooley Godward Castro Huddleson & Tatum, Five Palo Alto Square, 4th Floor, Palo Alto, CA 94306 (US).

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INFECTIOUS TARGETTED REPLICATION-DEFECTIVE VIRION

(57) Abstract

Novel methods and compositions are provided for introducing a *kill* gene, a gene capable of killing or inhibiting the growth of a cell into a specific target cell. Particularly, a virion which is substantially replication defective is prepared which has targeting capability for a specific cell type. The targeting may be provided by the native virion surface glycoprotein or the surface glycoprotein may be modified so as to comprise a member of a specific binding pair such as an antibody, or fragment thereof, to a tumor specific antigen, a viral protein expressed by a virally infected cell, or a receptor ligand. Thus the virion will target the normal target cell for the virus or a cell comprising the other member of the specific binding pair. The growth inhibitory capacity of the virion is provided by the *kill* gene incorporated into the virion.

+ See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic			SN	Senegal
CG	Congo	KR	Republic of Korea	SU	Soviet Union
CH	Switzerland	LI	Liechtenstein	TD	Chad
CM	Cameroon	LK	Sri Lanka	TG	Togo
DE	Germany, Federal Republic of	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

INFECTIOUS TARGETTED REPLICATION-DEFECTIVE VIRION

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of USSN 334,006, April 5, 1989 which is a continuation-in-part of USSN 326,195, filed March 20, 1989, which disclosures are hereby incorporated by reference.

INTRODUCTION

Technical Field

This invention relates to a binary vector, as well as methods for its preparation and use, comprising a packaging cell and a replication defective virion capable of introducing a cell growth inhibiting gene into a target mammalian cell.

Background

Traditional methods of cancer chemotherapy and treatment of a variety of viral diseases have often used systemic application of antineoplastic or antiviral agents. These agents have varying degrees of specificity for tumor cells or virus-infected as opposed to normal cells for inhibiting the growth of and/or killing of tumor cells or viruses within virus-infected cells. Thus, high doses of hormonally-related compounds such as hexestrol or cytotoxic agents such as methotrexate and nitrogen mustards have found use for treating cancer; drugs specific for inhibiting viral replication, such as AZT have found use for treating viral diseases. Unfortunately, the therapeutic index for these drugs tends to be narrow and the side effects of these treatments may be severe.

The ability to introduce a variety of genetic capabilities into mammalian cells, in particular to

introduce engineered genes into humans, opens up a broad vista of opportunity in the field of cancer chemotherapy and in the treatment of chronic viral diseases. Introduction of genes encoding toxins or other genes encoding potentially lethal products, collectively called kill genes, into a cancer cell or a virus-infected cell, would permit selective inhibition of growth or killing of the target cell with minimal effect on normal cells. Essential to the success of inhibiting growth of or killing of a specific target cell in vivo is the introduction of the kill gene into the target cell under conditions whereby the gene may be expressed.

By far the most efficient way of introducing such genes into cells is in the form of DNA or RNA encapsulated into virus particles. DNA and RNA as such is not taken up well by cells; by contrast, DNA and RNA in the form of virus particles is taken up very efficiently, mediated by receptors for the particular virus. Special cells have been constructed that allow the preparation of virus particles capable of mediating the transfer of genes to target cells. These cells supply virus proteins necessary for the assembly of virus particles. The genome, RNA or DNA, packaged into virus particles by these special cells, can contain any gene; in this application a kill gene. The genes encoding virus proteins have been removed from the packaged virus genome. Thus, the virus particles produced cannot replicate. The special cells, so-called packaging cells, have evolved for both DNA and RNA viruses from constructs requiring an additional virus, a helper-virus, to sophisticated cells no longer requiring helper-viruses, to support virus growth.

Replication deficient helper-free RNA tumor viruses produced by packaging cells have been used to transform cells derived from the human reticulo-endothelial system. These viruses and their packag-

ing cells have been derived from two strains of murine leukemia virus, Moloney murine leukemia virus (MoMuLV) that infects rodent cells, and strain 4070A that infects both murine and human cells. Human reticuloendothelial cells genetically engineered with retroviruses have been proposed for correction of enzyme defects: gene therapy. However, to stably introduce these genetically engineered cells into humans requires a bone marrow transplant. In preparation for a transplant, the patient must be given a lethal dose of x-irradiation in preparation for the graft, to remove the resident bone marrow cells. Thus a bone marrow transplant involves significant expense, and uncertain survival. Virus packaging systems have also been used to transfer genes into skin fibroblasts. Transformation of skin fibroblasts offers the advantage that engineered genes can be introduced into humans without the need for lethal whole body x-irradiation.

In the case of DNA viruses such as SV40, a papovaviruses, COS-7 cells have been used to construct helper-free, infectious, but replication defective SV40 virus (Gluzman, 1981). COS-7 cells contain an SV40 genome without the ori, or origin of replication. The cells produce SV40 T antigen, allowing for replacement of SV40 T antigen in the infecting virus genome with other desired genetic information. However, in the context of the present invention COS-7 cells are inadequate packaging cells. Early region SV40 gene replacement vectors (Tantigen replacement), expressed in COS-7 cells yield high levels of replication-deficient infectious helper-free virus, but also produce replication-competent infectious virus by homologous recombination with the endogenous viral genome. Late region SV40 replacement vectors (replacing VP1, VP2 or VP3), require helper virus to be packaged and infectious. Presumably the COS-7 cells are not capable of

sufficient expression of the late genes, VP1, VP2 and VP3 to yield infectious virus.

Also essential to the success of inhibiting growth of or killing of a specific target cell in vivo is the controlled expression of the kill gene in specific cell types. This is accomplished with tissue-specific enhancers/promoters. Enhancers/promoters are DNA elements that bind certain regulatory proteins only found in specific cell types. This binding leads to cell-specific transcription of genes. Enhancers/promoters have been described for B cells, T cells, liver cells, liver cancer cells, HIV-1 infected cells, and many other tissues/organs. Thus coupling of tissue-organ-specific enhancers/promoters to toxin genes can kill specific cell types.

While cell surface virus receptor interactions are frequently cell-specific, such interactions are not likely to be totally tissue/organ or cell specific. Thus it would be of interest to develop infectious replication-defective viral vectors with enhanced specificity toward specific cell types. This can be achieved by the combination of tissue-specific virus attachment, and tissue/organ-specific transcription of a kill gene. Helper-free virions produced by special packaging cells are constructed with properties aiding virion targeting to specific cell types. Further, kill genes under the transcriptional control of tissue specific enhancers/promoters, and encapsidated by the packaging cells, are constructed yielding vectors capable of toxin gene expression only in selected cell types. Thus therapeutics are produced with two levels of selectivity: cell-surface attachment, and time specific control of gene expression. These vectors possess enhanced specificity in killing of target cells such as virus-infected cells or cancer cells.

Relevant Literature

Production of retrovirus packaging cells capable of infecting human cells were made by incorporating the env gene from murine leukemia virus strain 4070A and the gag and pol genes from MoMuLV has been disclosed. These virus particles have been used to infect human reticuloendothelial cells, human hepatocytes and human keratinocytes. I.M. Verma, A. Miller and R.M. Evans, W08600922, filed July 29, 1985 and I.R. Morgan and R.C. Mulligan, W08700201 filed June 30, 1986.

Virus particles nonspecifically packaging host cellular nonviral RNA in the presence of helper viruses is described in Linial, J. Virol (1981) 38:380-382; Linial, et al., Cell (1978) 15:1371-1381; Watanabe and Temin, Proc Natl Acad Sci USA (1982) 79:5986-5990. Helper-free retroviruses produced from Moloney murine leukemia virus (MoMuLV) and avian reticuloendothelioses virus designed for murine and avian gene therapy have been disclosed by Mann, et al., Cell (1983) 33:153-159; and Watanabe and Temin, Mol and Cel Biol (1983) 3:2241-2249.

Helper-free packaged virus particles capable of infecting a wide variety of mammalian cells, including various human cells, have been described by Cone and Mulligan, Proc Natl Acad Sci USA, (1984) 81:6349-6353. A similar packaging cell line, was described by Miller, et al., Mol and Cel Biol (1985) 5:431-437.

Helper-free replication deficient RNA tumor viruses have been used to establish cell lines producing fibronectin (Schwarzbauer, et al., Proc Natl Acad Sci USA (1987) 84:754); Class II human major histocompatibility antigens (Korman et al., Proc Natl Acad Sci USA (1987) 84:2150); growth hormone (Morgan, et al., Science (1987) 237:1476); adenosine deaminase (Palmer, et al., Proc Natl Acad

Sci USA (1987) 84:1055); Factor IX (St. Louis and Verma, Proc Natl Acad Sci (1988) 85:3150); and phenylalanine hydroxylase (Peng, et al., Proc Natl Acad Sci USA (1988) 85:8146). Packaging systems have also been used to transform hepatocytes (Ludley, et al., Proc Natl Acad Sci USA (1987) 84:5335 and Wolff, et al., Proc Natl Acad Sci USA (1987) 84:3344) and skin fibroblasts (Morgan, et al. (1987) supra; St. Louis and Verma, (1988) supra; and Palmer, et al. (1987) supra).

Alterations to the 5'LTR region, enhancer sequences, "CAAT box" and "TATA box" in the long terminal repeats of the 5'LTR to eliminate uncontrolled and potentially tumorigenic transcription of DNA from the 5'LTR into the adjacent host-derived chromosomal DNA have been described by Yee, et al., Proc Natl Acad Sci USA (1987) 84:5197. Modification of the 3'LTR was described by Yu, et al., Proc Natl Acad Sci USA (1986) 83:3194-3198. Packaging cells in which the potential for recombination and recovery of infectious virus is reduced have been described by Danos and Mulligan, Proc Natl Acad Sci USA (1988) 85:6460-6464; Bosselman, et al., Mol and Cell Biol (1987) 7:1797-1806; and Markowitz, et al., J. Virol (1988) 62:1120-1124.

Packaging cells have also been isolated for adenoviruses (Shiroki and Shimojo, 1971; Graham et al., 1977; and Grodzicker and Klessig 1980). Using these packaging cells, host-range mutants of Ad5 (Harrison et al., 1977), deletion mutants of Ad5 (Jones and Shenk, 1979) ts mutants of AD12 (Shiroki et al., 1976), and adenoviruses vectors expressing foreign genes in high quantity (Berkner et al., 1987; Davidson and Hassell, 1987; Schaffhausen et al., 1987; Xhu et al., 1988) have been obtained. Similarly, packaging cells for Herpes Simplex virus have been isolated (Kimura et al., 1974; Maenab and Timbury, 1976). Using Herpesvirus packaging cells,

ts mutants of Herpes Simplex virus type 2 (Kimura et al., 1974; Macnab and Timbury 1976), and Herpesvirus expression vectors of foreign genes (Roizman and Jenkins, 1985; Thomsen et al., 1987) have been prepared.

Promoters and enhancers regulate genes that encode messenger RNA in mammalian cells. Promoters are located immediately upstream, 5', from the start site of transcription. Enhancers: increase the rate of transcription from promoters, act on cis-linked promoters at great distances, are orientation independent, and can be located both upstream, (5'), and downstream, (3'), from the transcription unit. Enhancers were first described for viruses. Later, enhancers inducible by hormones and metal ions and found only in specific tissues were described (Maniatis, T., S. Goodbourn, and J.A. Fisher. 1987, Science 236:1237-1245). Proteins synthesized only on one tissue type are frequently regulated by tissue specific enhancers. For example, actin and myosin in muscle; lens crystalline in the eye. For the tissue specific expression of kill genes, tissue-specific enhancers are of particular interest.

B cell and T cell enhancers are the best characterized tissue-specific enhancers. Immunoglobins are only synthesized in B cells. IgG heavy and light chain enhancers have been found within the introns of both the mu heavy-chain a kappa light-chain genes, each with a minimal functional region of about 140 base pairs (Gillies, S.D., S.L. Morrison, V.T. Oi, S. Tonegawa, Cell 33:717-728). These enhancers function only in B cells, not in T cells, colon cells, and other tissue types tested. The immunoglobin chain enhancers are functional in B cells because enhancer binding proteins, only present in B cells, activate transcription from genes cis to the enhancer. Similarly, the T cell specific T cell receptor (TCR) is only found on T

cells. Expression of the TCR is controlled by T cell enhancers: the alpha chain enhancer (Winoto, A., and D. Baltimore, 1989, EMBO J.8:729-733), and the delta chain enhancer (Redondo, J.M., S. Hata, C. Brocklehurst, and M.S. Krangel. 1990. Science 247:1225-1229).

Liver specific enhancers have been found for genes exclusively expressed in liver. These include: alphafetoprotein, transthyretin, alpha 1-antitryptsin, fibrinogen, and aldolase (Courtois, G., J.G. Morton, L.A. Campbell, G. Fourel and G.R. Crabtree, 1987, Science 238:688-692; Feuerman, M.H., R. Godbout, R.S. Ingram. and S.M. Tilghman. 1989, Molec. Cell Biol.9:4204-4212). Of particular interest in the Hepatitis B virus S antigen enhancer that is specific not only for liver cells but for liver cancer cells (Chang, H.K., B.Y. Wang, C.H. Yuh, C.L. Wei, and L.P. Ting, 1989, Molec. Cell Biol. 9:5189-5197; Chang, H.K., and L.P. Ting, 1989, Virology 170:176-183).

SUMMARY OF THE INVENTION

A novel replication-defective binary vector derived from an animal virus, conveniently a retrovirus or a papovavirus, together with methods for its preparation and use, is provided for introducing and expressing a kill gene in a mammalian target cell. The binary vector comprises (1) a packaging cell containing in its chromosome a nucleic acid sequence encoding a trans acting virus polypeptide required for the maturation of new virus particles and (2) a mobile genetic element comprising a modified genome in which at least one kill gene has replaced the trans acting virus function induced by the packaging cell. The modified genome of the mobile genetic element would contain a Ψ sequence or an ori for a retrovirus and a papovavirus, respectively. A modified ligand-receptor interaction introduced into

the mobile genetic element by expression of an altered virus surface receptor moiety gene in the packaging cell, or by covalent attachment of one member of a specific binding pair to the virion, is used to direct the mobile genetic element to the target cell. The packaging cell is grown to produce the mobile genetic element. The kill gene is transferred to the target cell directly by infection with the mobile genetic element. The packaging cells may produce the mobile genetic element in vivo to provide sustained release of the mobile genetic element which then infects the target cell. Tissue specific expression of the kill gene may be achieved by optional by introducing additional DNA sequences such as tissue specific enhancers/promoters to control expression of the kill genes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of the packaging cellpCRIPenv-.

Figure 2 shows the structure of the MoMuLV provirus in pNC1.

Figure 3 shows the structure of the Nde1-Nsi1 insert of pUC19-env-.

Figure 4 shows the structure of plasmids pNCSH-1, pNC-SH-2, etc.

Figure 5 shows the structure of pNC-CD_4.

Figure 6 shows the three constructs having a second copy of gag, pol and env.

Figure 7 shows the structure of pNC-HSV-tk(2).

Figure 8 shows the structure of pNC-HSV-AS.

Figure 9 shows the structure of pNC-HSV-X, where X is a protease, ribonuclease or DNAse.

Figure 10 shows the genetic map of SV40.

Figure 11 shows the three constructs for VP1, VP2 and VP3.

Figure 12 shows the structure of pNCHSVkill.

Figure 13 shows the structure of pNCSV1.

Figure 14 shows the structure of the Diphtheria A tox insert.

Figure 15 shows a schematic representation of the SV40 and LPV genomes.

Figure 16 shows the structure of PNCLV2, and LPV Vector.

Figure 17 shows the structure of a production minichromosome.

Figure 18 shows the structure of a T cell enhancer/promoter Dyphtheria A toxin.

Figure 19 shows the structure of the HIV-1 enhancer/promoter Dyphtheria A toxin.

Figure 20 shows the structure of the Hepatitis B virus enhancer/promoter Dyphtheria A toxin.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

A novel binary vector, and methods of preparation and use, is provided for the introduction into a specific mammalian host cell, particularly a human cell, of a kill gene capable of transcription and expression in said host cell. The binary vector comprises a packaging cell and a mobile genetic element. The host range for cell attachment of the vector is determined by modification of the cell surface ligand-receptor interaction of the mobile genetic element of the vector. Modifications in cell attachment can be accomplished by incorporation of a modified virus surface receptor moiety gene, such as a retrovirus env gene or the VP1 gene of a papovavirus into the packaging cell. Alternatively, the virion surface receptor moiety may be coupled to a targeting means comprising one member of a specific binding pair (sbp) such as an antibody to a cell surface antigen or a peptide hormone so that the vector binds to the cell surface antigen or peptide hormone receptor which comprises the other member of the sbp on the target cell.

Inhibition of the growth of or killing of the target host cell, is provided by introduction of a nucleic acid sequence encoding a kill gene, such as a toxin gene, an anti-sense transcript or other trans acting regulatory element. In addition, the vector may include a marker that can be used for detection of the vector or fragments thereof in the target cell by screening or selection. The packaging cells are grown to produce the mobile genetic element containing the minichromosome which is used to infect target mammalian cells under appropriate conditions for transfer of the minichromosome containing the nucleic acid sequence of interest into the target cell. When the genotype or phenotype associated with the kill gene is expressed, the result is inhibition of cell growth or killing of the target cell.

Tissue specific expression of the kill gene further narrows the specificity of target cell killing. Tissue specific gene expression is achieved by the use of highly specific enhancers/promoters; that is DNA sequences to which are bound proteins that only exist in certain types of cells. These proteins are activators of transcription that regulate the expression of certain genes that are therefore expressed only in these cells. The existence of tissue/organ-specific enhancers/promoters provides the opportunity for targeting anti-cancer and anti-viral agents to the specific organ or cell type where disease has arisen. This is done by (a) introducing into cells genes that encode toxic or potentially toxic proteins, and (b) controlling the expression of such genes with highly specific enhancers/promoters. Thus cells of one particular organ, cells with a receptor for the virus, and cells with the specific activators, for example specific enhancers/promoters, will be killed.

An object of the invention is to produce a binary vector capable of transmitting antiproliferative or cytotoxic genetic material, which will be referred to generally as a kill gene, in a one-way nonreversible manner to a specific target cell. By kill gene is intended a nucleic acid sequence which provides for inhibition of cell growth or cytotoxicity in any of a variety of ways, including the following. The kill gene may affect an essential cell function directly, for example, by providing for synthesis of an anti-sense transcript of an essential cell component or of an oncogene. The kill gene may also encode a polypeptide capable of directly inhibiting cell growth such as Diphtheria toxin or one which may catalyze formation of an inhibitory or toxic compound, such as, for example Herpes virus thymidine kinase (HSV-tk) which is part of a cascade to form acylo-GTP from acyclovir. In the latter instance, when acyclovir is administered, the antiviral agent acylo-GTP is formed.

The binary vector consists of: (1) a packaging cell encoding a trans acting polypeptide(s) required in the maturation of new virus particles, and (2) a mobile genetic element comprising an infective yet replication incompetent virion and a minichromosome. The binary vector is prepared as follows. A virus gene encoding the trans acting viral function(s) is removed from the wild-type virus genome and inserted into the chromosome of a packaging cell that is otherwise permissive for the virus. By "packaging cell" is intended a genetically constructed mammalian tissue culture cell that produces the necessary virus packaging proteins. The genetic material for the virus packaging proteins is not transferred with the mobile genetic element, hence the virus cannot replicate. The virion produced by the packaging cell, the mobile element of the binary vector, contains a modified genome or minichromosome in which

genes designed to inhibit the growth of, or to kill, the target cell have replaced the trans acting function that has been incorporated into the chromosome of the packaging cell.

Packaging cells may be any animal cells permissive for the virus, or cells modified so as to be permissive for the virus; or the packaging cell may be one which is made permissive for the proviral construct, for example, with the use of a transformation agent such as calcium phosphate. Cell lines which can be used as packaging cells for retroviruses include rodent cells such as NIH/3T3 cells and other murine cells; a suspension cell line such as Chinese Hamster Ovary cells (CHO) or L929 cells; for papovavirus, cells such as BSC-1 or VERO cells from African Green Monkeys, or human cells, such as WI38; and for adenoviruses, KB cells and HeLa cells. Of particular interest as packaging cells are cells, particularly human cells, deficient in glucocorticoid receptors. In some instances, it may be desirable to immortalize normal human cells by transfection with the polyoma virus T antigen, and to adapt the culture to serum-free suspension culture.

To prepare the packaging cell capable of producing the desired replication-defective mobile genetic element, at least a first and a second construct will be transformed into a packaging cell, the constructs being as exemplified below for a retrovirus. The first construct, the mobile genetic element, is prepared having the following components in the order of transcription:

5'LTR--P.S.--(V.G.)0-2--G.I.--(V.G.)0-2--3'LTR

wherein 5'LTR and 3'LTR have their usual meaning; P.S. intends a viral packaging sequence; V.G. intends a trans acting gene, and G.I. intends a kill gene and wherein the components of the construct are operably linked.

A second construct, and optionally third and fourth constructs, used to construct the packaging cell, comprises the following components in the direction of transcription, a transcriptional initiation region, at least one of a gag, pol and an env gene and a transcriptional termination region, wherein the components are operably linked. The general structure of these constructs is as follows:

$$(5'\text{-LTR})_a\text{--}(P)_b\text{-}\ (V.G.)_c\text{--}T.R$$

wherein 5'LTR and V.G. have the meaning described above; P intends a promoter other than the 5'LTR; T.R intends a translational termination region, a polyadenylation signal and a transcriptional terminator; a, b and c are integers, a and b being from 0-1 where the sum of a + b is at least 1, and c is 1-3, usually 1-2. V.G. additionally may comprise a single copy or a plurality of copies of the same trans acting gene, for example, gag gag or pol pol. Thus, generally the second construct will provide at least one copy of each of the viral gag and pol genes without the packaging Ψ sequence and the 3'LTR. A third construct will comprise a modified env gene without the Ψ sequence and 3'LTR. Unless already provided, a fourth construct will provide at least one copy of the gag or pol gene, without the Ψ sequence and the 3'LTR. The desired replication defective virion produced by the packaging cells will have a genome containing the 5'LTR and Ψ sequences, a kill gene and the 3'LTR. The constructs which provide the Ψ packing sequences and the gag, pol and modified env genes may be derived from any animal retrovirus, particularly Moloney murine leukemia virus, an avian leucosis virus, or an HTLV virus.

Analogous constructs to those described above can be prepared for binary vectors employing papovavirus, where gag, pol and env are replaced with T-antigen VP1, VP2 and VP3. P, the promoter is preferentially an inducible enhancer/promoter such

as the metalothionein promoter, or murine mammary (MMTV) promoter.

In order to obtain substantially replication-defective virions potential homologous recombination reconstruction of infectious virus must be eliminated. Thus, to obtain production of substantially replication-defective virions by the packaging cells, the preferred method for preparation of the packaging cells is to introduce at least two mutant viral genomes into the packaging cell, most preferably three. This containment scheme will effectively prevent homologous recombination leading to reconstruction of infectious virus. The probability of recombination occurring if two trans acting virus genes are used is less than 1 x 10-14; with the use of three separate mutant virus genes, the probability of recombination reconstruction of infectious virus is less than 1 x 10-21. For retroviruses, in the most preferred method of construction of the packaging cell, the gag, pol and env genes are transformed into the packaging cell on three separate plasmids, preferably sequentially, in any order, by any convenient means, for example, by the calcium phosphate precipitation protocol. For DNA viruses, vectors such as those prepared from papovavirus and patterned after SV40, the SV40 ori, with or without the large T antigen could be maintained in the mobile element of a binary vector. T antigen can also be supplied by the packaging cell, but the ori must be present in the mobile genetic element. If T antigen is present in the mobile genetic material, the minichromosome will replicate to high copy number in the target cell, increasing expression of the kill gene(s). If the T antigen is absent from the minichromosome, the minichromosomes will not replicate in target cells. papovavirus vectors patterned after SV40 are episomal and transitory in nature. Therefore, the kill genes encoded by the

minichromosomes must be sufficiently lethal to kill the cell in an active manner. This restricts the kill genes to activities such as RNAse, DNAse, and protease. Since these vectors are transitory, they are particularly attractive for use in humans as they do not permanently genetically engineer the host cell.

In order to produce high titer virions, high producing packaging cells are identified by screening the transfectants, for example by immunoassay, by PAGE, and the like, for packaging cells making quantitatively more gag, pol and env, or T antigen VP1, VP2 and VP3 expression products. Once the high producing packaging cells have been identified, they may be transfected using the provirus construct comprising the kill gene. The resulting transfectants may be selected, as described above, and the so-obtained packaging cells grown to express the viral genes and produce the desired substantially replication-defective virions. The object is to produce a binary vector capable of transmitting genetic material to the target cell in a one-way nonreversible manner and for the gene of a trans acting polypeptide to be incorporated into the chromosome of a host cell otherwise permissive for the virus. The chromosomally integrated gene(s) within the packaging cell must not be capable of being mobilized into super-infecting viral genomes, yet the minichromosomes must be capable of producing infectious virus.

Analogous vectors can also be derived from DNA viruses such as Adenovirus type, Herpesvirus, and Papovaviruses, such as SV40, lymphotropic papovavirus (LPV), BK virus (BKV) and JC virus (JCV). For the papovaviruses, the minichromosome (the mobile DNA) packaging into the mobile genetic element, also called pseudovirions, the minimum requirements for a construct are an ori and a circular genome within

about 75-102% of 5243bp. In the case of SV40, COS-7 cells can complement necessary trans acting genes, allowing for construction of helper-free, infectious, but replication defective SV40 virus (Gluzman, 1981). COS-7 cells produce SV40 T antigen, thus the SV40 T antigen in the virus genome can be replaced with other genetic material. In particular, the genome and distribution of trans acting genes of SV40, LPV, BK and JC viruses is well established. Thus, site specific mutagenesis to affect the cell type of virus attachment as controlled by the outer capsid proteins could be readily evaluated.

DNA viruses, in particular papovaviruses, may offer advantages over retroviruses as a source of mobile genetic material. The best studied DNA virus, SV40, has many desirable features that would ease virus purification, chemical coupling procedures, manufacturing and quality control: the virus grows to titers of 10^8 to 10^9 pfu/ml in packaging cells such as COS-7 and CMT4; the virus is stable at 4° C; the infectivity of the virus withstands harsh treatment such as deoxycholate and cesium chloride; fully packaged infectious virions can be readily isolated from defective empty capsids in cesium chloride; the virus does not cause disease in humans; SV40 minichromosomes are invariably lost from infected cells without positive selective pressure; T antigen mutants that are transformation(-) yet replication(+) can be used to support SV40 minichromosome replication avoiding concerns of T antigen induced tumors; SV40 minichromosomes are genetically stable; and cell killing genes can be controlled by the early SV40 promoter which is active in many cell types or, alternatively, the HIV-1 LTR and T-cell receptor enhancer/promoter, which are both highly T-cell selective, the IgG heavy-chain enhancer/promoter which is highly selective, or the

hepatitis B virus surface antigen enhancer/promoter which is highly hepatoma cell selective.

In considering ablative virus therapy, the transient expression of papovavirus minichromosomes represents a significant improvement in the concerns involved in genetic engineering of humans. The goal in ablative virus therapy is to specifically affect a cell type without side effects or lasting residual genetic interference with the human chromosome. If the amphotropic retroviruses deliver proviruses to and transform the majority of human cells encountered, this goal is not accomplished. Many cells are needlessly permanently genetically engineered. With the use of, for example, papovavirus minichromosomes patterned after SV40, the tissue specific expression of genes can still be accomplished, yet no permanent genetic engineering of nontarget cells would occur. The viral therapeutic is metabolized and dilutes out in time as any conventional pharmaceutical product.

The host range of a virus generally is partially determined by a portion of the virus surface receptor moiety on the surface of the virion. Virus host range is further defined by the unique molecular biology of the infected cell by enhancers/promoters controlling gene expression. Some viruses attach to a specific cell type. The virus produced by the packaging cell will also attach to a specific cell type, by use of a natural surface receptor moiety; it can also attach to cells using a receptor moiety which has been altered. For example, a glycoprotein receptor moiety may be provided, the receptor comprising one member of an sbp: The glycoprotein gene can be modified so that the produced virion may be directed to a specific cell type. Specific amino acid sequences such as, for example, the CD4 sequence that recognizes HIV gp120 can be fused to the env gene of a retrovirus or the VP1 gene

of a papovavirus using methods known to those skilled in the art. Gene fusions comprising heavy chain antibodies for the F(ab')2 fragment of a tumor-specific antigen fused to the constant region of the env gene or the VP1 gene can also be used as a targeting means. The fused gene can be inserted into an appropriate plasmid for transformation into the packaging cells. In this case the specific antibody light chains must also be cloned into the packaging cell.

In addition, the virus surface receptor glycoprotein can be modified for example, by introducing a mutation to give a lysine, histidine, or cysteine residue in the protruding portion of the glycoprotein yielding an amino group, a histidine free for coupling by organic chemical means, such as a sulfhydryl group. Cell-specific reagents such as F(ab')2 fragments of specific antibodies such as those to pancreatic cancer or stomach cancer, other tumor-specific antigens or membrane receptors can be coupled with glutaraldehyde, periodate, or maleimide compounds, or by other means known to those skilled in the art to the altered virus. Such couplings may also be made directly to wild-type or unmodified virus env gene glycoprotein products, where coupling can be to a carbohydrate moiety, a sulfhydryl group, an amino group, or other group which may be available for binding. Examples of other ligands which can be coupled by this method include the antibody to HIV gp120 to target HIV infected lymphocytes, soluble CD4 protein to target HIV infected cells, transferrin to target transferrin receptors, and other members of sbps such as gastrin, secretin or cholecystokinin to target their respective cell receptors.

A preferred method of coupling the targeting means to the env gene or VP1 gene encoded glycoprotein is to couple sulfhydryl groups of the hinge region of an antibody to the retrovirus

envelope glycoprotein or the papovavirus VP1. For coupling see, for example, Ishikawa, et al., J. Immunoassay (1983) 4:209. Other coupling groups include carbohydrate couplings and amino group couplings. In the case of an antibody, or the immunoreactive Fab' or F(ab')2 portions of an antibody, particularly a monoclonal antibody (MAb) could also be coupled directly to the surface of the virus particle using methods well known to those skilled in the art. Once the virion-MAbs conjugate reaches the endosomal compartment of the target cell, the virion must proceed into the normal virus infection pathway. The covalently attached MAb may or may not hinder these processes.

It is also possible to target a specific cell type by using the natural selectivity of a particular virus. For example, several papovaviruses exhibit a narrow host range. LPV infects only lymphocytes. Similarly, BKV infects only kidney and liver cells. JCV only infects glial cells.

LPV could be a candidate to deliver a mini chromosome to B lymphocytes. With the use of k chain of Igh μ chain enhancers, LPV derived kill vectors can be envisioned to treat B cell disorders such as B cell leukemia. Recently, a mutant of LPV has been isolated that also replicates in T lymphocytes. (Kanda, et al. J. Virol (1985) 55:96-100). This mutant LPV, LPV-76, has been sequenced, and the change is responsible for extending the host range of LPV-76 are three amino acid changes in the VP1. (Kanda, et al. J. Virol (1986) 59:531-534. In the case of AIDS, the use of LPV-76 or more specifically the VP1 of LPV-76 would direct the virus vector exclusively to B and T lymphocytes. The use of the tat responsive HIV-1 LTR would provide a 1,000-fold enhancement of transcriptional activity in HIV-1 infected cells as compared to non-HIV-1 infected cells of an appropriately designed transient

expression minichromosome. Thus, AIDS and lymphocytic disorders represent therapeutic targets of virus vectors designed as therapeutics.

Virus vectors containing minichromosomes can be developed using a papovavirus such as SV40 as follows. An SV40 early replacement vector would be designed where a toxic gene would be controlled by the tat responsive region TAR of the 5'-HIV-1 LTR. Thus this region would be transcriptionally silent in cells lacking the 14.5 Kd tat protein. Indeed the HIV-1 LTR contains binding sites for nuclear factor activated (NFAT-1) (Shaw, 1988), negative regulatory factor (NRF) (Ratner, 1988), Nuclear factor kb (NFkB) (Nabel, 1987; Tong, 1987), Sp1 (Jones, 1986), TATA-box transcription factor (TFIID) (Garcia, 1987), leader binding protein (LBP) (Jones, et al. Genes Development 2:1101-1114 (1988), and CCAAT transcription factor (CTF) (Jones, et al. (1988) supra), and CCAAT transcription factor (CTF) (Jones, et al. (1988) supra; for review see Peterlin, et al. (1988) Molecular Biology of HIV, AIDS 2: (suppl. 1): 529-540. TAR found in mRNA transcribed from position 0 to +480 within the HIV-1 LTR responds to the HIV-1 tat regulatory protein.

The combined action of the 14.5 Kd tat protein TAR stabilizes and transports mRNA transcripts resulting in a 1000-fold enhancement of gene expression. Use of the HIV-1 LTR -350 to +80 to control toxin expression yields an kill vector specifically active in HIV-1 virus infected cells.

Minichromosomes, in which the major features are an SV40 ori with SV40 late functions intact, the HIV-1 5'LTR, and a kill gene driven by the HIV-1 5'LTR, can be prepared in COS-7 (Gluzman, Cell (1981) 23:175-182) or CMT4 (Gerard, et al. (1985) Molec. Cell Biol. 5:3231-3240) cells. COS-7 cells are constitutive for wild type T antigen and VP1, VP2, and VP3 in trans. CMT4 is inducible for wild-

type T antigen by the metallothionein promoter. The HIV-1 5'LTR should be transcriptionally silent in CV1 or VERO cells, or the cells used to construct the papovavirus packaging cells. Thus the kill gene can include not only the nontoxic HSV-tk gene product but more aggressive mechanisms such as angiogenin, a 14,000 dalton ribonuclease encoded by 369 bp of DNA (Kurachi, et al. (1985) Biochemistry 24:5494-5499), and the ricin or Diphtheria toxin A chain genes. In the case of a more aggressive kill gene product such as angiogenin, the ricin A chain or the Diphtheria A chain and if the HIV-1 5'LTR construct is not adequately transcriptionally silent in the packaging cell construct, an angiogenin inhibitor (Lee, et al. (1988) Biochemistry 27:8545-8553) or an antisense RNA could be used to protect the packaging cell from the toxin cell mutants resistant to the toxin can also be constructed. These inhibitors or a mutant resistant genes could be readily introduced with an amphoteric retrovirus thereby protecting the packaging cell from unwanted toxin gene expression.

It is also necessary to show that recombination does not produce replication competent virions. Minichromosomes encapsidated by COS-7 cells have produced infectious virus by recombination. This has not been a concern as SV40 has not been proposed for human therapy. Work with the retrovirus packaging cells, psi-2, psi-AM, and PA-317 has also shown infectious virus by recombination. This recombination has been shown to require homologous sequences, and occurs at a rate of about 1 x 10-7. As discussed previously, separation of the three MuLV trans packaging factors gag, pol, and env into two constructs drops the appearance of recombinant infectious virus below detectable levels. Probability says that the chance of two independent homologous recombination events each with a probability of 1 x 10-7 is 1 x 10-14. This is an

undetectable frequency. Thus the development of new packaging cells with two separate insertions of trans functional virus proteins will be required (Gluzman, 1981) supra; Danos, et al. (1988) Proc. Nat. Acad. Sci. USA 85: 6460-6464).

Specifically a new construct in CV1, CV1P, or VERO cells that includes a mutant SV40 T antigen that is SV40 ori replication positive but transformation negative, and also contains in a separate cellular chromosome integration site for one of the capsid proteins, perhaps VP2 or VP3 would solve this issue. Using these constructs, a nonamplifiable SV40 minichromosome can be delivered to a target cell by an SV40 capsid, and lymphocyte and HIV-1 infected cell specific transcription will occur. This transcription will encode a function designed to kill the target cell.

A disadvantage of a strategy that does not include T antigen in the minichromosomes is the lack of transient amplification of the infecting minichromosome. This could result in a shortfall of desired toxicity in the target cell. To include an SV40 ori and a replication enabling T antigen would solve this problem. Minichromosome DNA would be transiently amplified, gene products would be transiently amplified, and enhanced toxicity could be achieved. However, the cell transforming and cell immortalization capabilities of SV40 T antigen must be addressed.

SV40 T antigen has long been known to be responsible for cell transformation in culture, and cell transformation has been associated with tumorigenicity in animals (Osborn, et al. J. Virol. (1985) 15:636-644); Kimura, et al. Proc. Nat. Acad. Sci. (USA) (1975) 72:673-677. The concerns relating to use of SV40 T antigen can be reduced by the use of mutant T antigens deficient in transformation capability.

SV40 T antigen is a multifunctional 708-amino-acid phosphoprotein. Extensive mutational analyses of large T antigen has demonstrated that discrete regions of this multifunctional protein mediate these varied functions (Mohr, et al. J. Virol. (1989) 63:4181-4188); see Livingston, et al. Mol. Biol. Med. (1987) 4:63-80) for review).

The transforming activity of T antigen is located in the N-terminal fifth of the protein (Sompayrac, et al. Virology (1988) 163:391-396). The T antigen stabilization of p53 nonviral tumor antigen in mice, while a logical candidate for the mechanism of T antigen induced cell transformation, is not required for transformation by SV40. Instead the Rb binding site seems to be involved. The Rb susceptibility gene, which when deleted or inactivated predisposes the development of retinoblastoma (Friend, et al. (1986) supra) binds SV40 T antigen (DeCaprio, et al. (1988) 54:275-283). Substitution and internal deletion mutants of T antigen amino-acid residues 105-114 yielded a protein that failed to complex with the Rb susceptibility protein and failed to induce transformation as measured by focus formation and growth in soft agar. Thus mutant SV40 large T antigens that support minichromosome replication but not cellular transformation by virtue of mutation in AA 105-114 can be incorporated into minichromosome constructs.

As described in the construction of packaging cells for a nonamplifiable SV40 minichromosome therapeutic, another packaging cell construct is required for amplifiable minichromosome constructs in order to eliminate the possibility of recombination between mutant T antigens and the wild-type T antigen found in COS-7 and CMT4 cells. In this case the CV-1 or VERO derived cells would only contain capsid proteins. The packaging cells for amplifying minichromosome constructs will contain the SV40 late

region of VP2 and VP3 on separate constructs. Some of the constructs can be based on LPV, BKV and JC viruses, viruses closely related to SV40. Titers of about 1 x 10^9/ml can be expected from such a packaging cell where the virion proteins are driven by the metallothionein promoter (Gerard, et al., Moll. Cell Biol. (1985) 5:3231-3240); Settleman, et al. Proc. Nat. Acad. Sci. (USA) (1988) 85:9007-9011).

Cell specific infection. VP1 from LPV and BKV, and enhancers from LPV, BKV, the T-cell receptor, and hepatitis B virus useful in targeting lymphocytic diseases and liver cancer respectively may also be prepared. An HIV-1 therapeutic is dependent on the known activated lymphocyte transcriptional activators, and the known HIV-1 tat gene product. To develop ablative virus therapy into a more universal therapeutic approach will require specific knowledge of the target cell nuclear transcriptional activator factors(s) environment, or to target the virus to specific cell types through specific receptor interactions. Recently it has been reported that SV40 binds to protease and carbohydrate sensitive receptors at the apical surface of cells (Clayson, (1988) [reference]; Clayson, et al. J. Virol. (1989) 63:1095-1100) and specifically binds to the class I major histocompatibility complex protein (Atwood, et al. J. Virol. (1989) 63:4474-4477). The polypeptide sequences presumably on VP1 responsible for the SV40 virus cell receptor interaction are unknown.

The receptor interaction of several other viruses is known in considerable detail (Crowell, et al. in Virus Attachment and Entry Into Cells (Eds. Crowell and Lonberg-Holm) Amer. Soc. for Microbiology (1986) pp. 1-9). The best studied is the gp120:CD4 interaction of HIV and simian immunodeficiency disease (SIV). Recently the cellular receptor for rhinoviruses has been shown to be ICAM-1, another immunoglobin supergene family member (Staunton, et

al. Cell (1989) 56:849-853; Greve, et al. Cell (1989) 56:839-847). The putative virion surface binding site for picornaviruses, the canyon receptor, is also highly conserved (Colonno, et al. Proc. Natl. Acad. Sci. USA (1988) 85:5449-5453; Rossman, et al. Virology (1988) 164:373-382).

Since SV40 infects most human cells, and LPV infects B lymphocytes attachment of a receptor protein to SV40 capsids could selectively direct the virus to target cells. Perhaps binding soluble CD4 would direct SV40 or LPV to HIV-1 infected lymphocytes. An antibody fragment could also direct virions to p41 or gp120 representing cells much like an antibody attached to a toxin has been used to selectively kill HIV-1 infected cells in vitro (Chaudhary, et al. Nature (1988) 355:369-372).

Receptor-mediated attachment to a cell of various ligands such as transferrin, monoclonal antibodies, and many viruses, is followed by transport of the receptor-ligand complex into a coated pit. The coated pit invaginates and buds off the cell membrane into an internal vesicle. (Pastan and Willingham Science (1981) 214:504-509). The internal vesicle then fuses with an endosome. The fate of the endocytosed material in an endosome is diverse. In the case of transferrin, the pH environment of the endosome causes a conformational shift, releasing Fe+++. The transferrin and transferrin receptor are then recycled to the cell surface for further use. If a MAb to the transferrin receptor is coupled to the ricin A chain, 50% of the MAb-ricin conjugate is recycled to the surface and released into the medium. However, viruses pass through the endosome apparently intact in the case of SV40 and adenoviruses, but in the case of influenza virus, the low pH within the endosome triggers an irreversible conformational change in the hemagglutinin (HA) which renders the HA molecules

fusogenic. The mature form of HA consists of two subunits HA1 and HA2. The conformational change uncovers the amino-terminal hydrophobic domain of HA2 which, at neutral pH, is buried within a trimer of HA. With some variations, similar mechanisms have been demonstrated for the entry of alpha-, rhabdo- and flaviviruses, and some murine RNA tumor viruses; All bind to cell surface components of the target cell membrane, are internalized into endosomes and undergo low pH-dependent fusion (Simons et al. (1982) Sci. Am. 246: 56-66).

The use of MAb directed viruses has the advantage of making use of natural adaptations of viruses to survive and pass through the endocytotic pathway. Where the proteolytic enzymes found in endosomes play a role in virion uncoating, it may be of use to include an amino acid sequence providing for proteolytic cleavage of the targeting portion of the virus in the endosomal vesicle by, for example, a cathepsin or a peptidase. Cathepsin D, a preglysosomal enzyme found in endosomes, cleaves between -Phe-Phe-. The adjacent amino acids are not important. (Blum and Cresswell Proc. Nat. Acad. Sci. USA (1988) 895:3975-3979; Diment and Stahl, J. Biol. Chem. (1985) 260:15311-15317). Therefore, a peptide bridge between, for example, a $F(ab)'_2$ fragment and the virion, cleavable in the endosomal environment, would be $(AA-_n-Phe-Phe-(AA)_m$ where n + m is not more than about fifteen. As an example, monoclonal antibodies conjugated to methotrexate have been disclosed by Umemoto et al. Cancer Immunol. Immunother. (1989) 28:9-16.

Additionally, enhanced release of the targeted virion from the targeting moiety may be achieved by exploitation of the low pH environment of the endosome. In a strictly chemical environment, disulfide bonds are reduced at pH 5.5 in the presence of reducing agents. If in addition to the low pH of

endosomes, the endosomal environment is also a reducing environment, disulfide bonds may be labile in endosomes. Thus use of a disulfide bridge between the $F(ab')_2$ fragment or other targeting moiety and the virion may provide for a bond which is labile in the endosome, thus releasing the virion.

Early claims of a "perfect" monoclonal antibody against all tumors, or even a tumor-specific antibody against all tumors of a given cancer have been discounted. However, MAbs having high affinity binding ("specific" binding) with tumor cells and minimal or restricted non-specific binding ("non-specific" binding) with normal adult cells have been shown to be useful for radioimaging of melanoma and with carcinomas of the breast, colon, and rectum. Also, nonspecific MAb cytotoxicity becomes evident only at high antibody concentrations, a problem necessitated by the low inherent cytotoxicity for the target cell or the need for many MAb-cell surface interactions to produce a radioimage.

This non-specific cytotoxicity is accentuated by the current therapeutic strategies that use the whole antibody molecule. Fc fragments have been retained in the MAb preparations to increase the circulating half-life of the antibody, to provide convenient chemical coupling sites for conjugate preparation, and to recruit Fc receptor mediated cytotoxic T cell killing. However, retention of the Fc fragment also increases Fc receptor mediated binding to normal cells, and contributes to the strong humoral immune response that limits in vivo immune therapy to 2-4 I.V. injections. The immune response to the $F(ab')_2$ fragment has been shown to be minimal. The disadvantage of $F(ab')_2$ therapy is the short half-life of the fragment in vivo but by attaching $F(ab')_2$ to a solid phase (such as a virion) the half-life and immunogenecity problems of current MAb therapy can be minimized.

MAbs, with or without attachment to therapeutic drugs, have been shown to be cell specific in vivo in gastrointestinal malignancies and leukemia/lymphoma. Epenetos et al. The Lancet (1982) 2:999-1004. In addition, immunohistochemical typing of tumor biopsies at various stages of malignant progression of tumor, with a panel of MAbs, has confirmed the existence of antigenic heterogeneity within a given tumor type. This has generated information on: (1) early detection of micro-metastases, (2) discrimination between benign and malignant lesions, and (3) tumor prognosis (Aukerman et al. (1987) In: Oldham, R.K., (ed) "Principles of Cancer Biotherapy", Raven Press, NY pp 21-47). From a therapeutic viewpoint, the need for a "cocktail" or mixture of several MAbs has been recognized. Use of cocktails comprising a plurality of MAbs, usually two to five MAbs, recognizing different antigenic determinants has substantially improved the percentage of a given tumor type recognized in a heterogeneous patient population. Liao et al. Cancer Immunol Innunother (1989) 28:77-86.

For use with diseases such as AIDS and primary hepatocellular carcinoma, the specific interactions of gp120 and CD4 and MAbs directed against Hepatitis B virus surface antigen (HBsAb) can be used. However, in cancers with antigenic heterogeneity, this issue can preferentially be addressed by use of a cocktail of MAbs. In coupling of MAbs to direct the host range of a virus infection, this cocktail, comprising a plurality of MAbs directed to different epitopic sites can be premixed prior to the coupling reaction of MAbs and virions. Alternatively, since the therapeutic virions containing the kill genes can all be identical, the mixture can be prepared after coupling.

A DNA sequence encoding the kill gene of interest can be synthesized employing conventional

techniques such as overlapping single strands which may be ligated together to define the desired coding sequence. The termini can be designed to provide restriction sites or one or both termini may be blunt-ended for ligation.

Alternatively, the kill gene may be isolated by various techniques. For example, mRNA can be isolated, the mRNA reversed transcribed, and the resulting single-stranded (ss)DNA used as a template to prepare double-stranded (ds)DNA and the (ds)DNA gene isolated. Another technique is to isolate a piece of the DNA, and using a probe, appropriately degenerate, comprising a region of the most conserved sequences in the gene of interest, identify the sequences of interest. The probe can be considerably less than the entire sequence, but should be at least 10, preferably at least 14, more preferably at least 20 nucleotides in length. Longer oligonucleotides are also useful up to the full length of the gene of interest. Both DNA and RNA probes can be used.

Kill genes of interest include the HSV-tk gene, and genes encoding RNAase, protease, and DNAase. Other kill genes which may find use include anti-sense RNA directed at, for example, human oncogenies such as H-ras, K-ras, or N-ras genes. The anti-sense RNA will generally be about 5 to 100 bp in length, preferably about 20 bp in length. Thus, for example, anti-sense RNA can be synthesized to various functional sites of the HIV genome (see, e.g., Gutch & Liddell (1988). Anti-sense oligomers complementary to sites within or near the HIV sequences repeated at the ends of the retrovirus RNA (R region), to splice acceptor, and splice donor sites, find particular use.

In terms of transcriptional termination, RNA cleavage and polyadenylation regions provide for proper maturation for the mRNA transcripts and are necessary for efficient expression. The native 3'-

untranslated region may suffice, but the polyadenylation signal from, for example, SV40, particularly including a splice site which provides for more efficient expression, could also be used. Alternatively, the 3'-untranslated region derived from a gene highly expressed in a particular cell type (for example, Ig in myeloma cells) could be fused with the gene of interest. The particular termination regions are not critical to this invention, being primarily for convenience, so long as they provide for expression of the gene of interest.

After each manipulation of the nucleic acid sequence of interest, and the development of the nucleic acid constructs, the resulting plasmids will be cloned and isolated and as required, the particular components of interest analyzed as to which sequence has been developed to ensure that the proper sequence has been obtained. Depending upon the nature of the manipulation, the desired sequence may be excised from the plasmid and introduced into a different vector or the plasmid may be restricted and the desired component(s) manipulated, as appropriate.

In some instances, a shuttle vector will be employed where the vector is capable of replication in different hosts requiring different replication systems. This may or may not require additional markers which are functional in the two hosts. Where such markers are required, these can be included in the vector. The plasmid containing the cassette, two replication systems and the marker(s) may be transferred from one host to another as required. For selection, any useful marker may be used. Desirably, resistance to an antibiotic or methotrexate is of interest. However, although a marker for selection is highly desirable for convenience, other procedures for screening transformed cells have been described. For example, transformed cells may be screened by the

specific products they make, for example, by immunological or enzymatic methods. The various constructs of interest will then be purified.

The virions produced by the packaging cells and comprising the kill gene of interest and flanking regions providing for regulation of expression may be introduced into the intended host by any convenient means, such as intravenous or subcutaneous injection, and oral or intranasal administration. Alternatively, in the case of retrovirus, the packaging cells themselves may be introduced into the host by grafting, for example, skin cells, and the like, where the cells grow and express the viral genes and produce the desired virions directly in the host.

The subject compositions can use a wide variety of toxins as kill genes. Genes which may be used include kill genes and anti-sense RNA. The HSV-tk gene is a preferred cytotoxic gene in retrovirus constructs. A specific retrovirus cell-targeting vector to deliver the HSV-tk gene to the target cells can be developed. The target cells can then be killed after administration of acyclovir which is phosphorylated by HSV-tk but not by human thymidine kinase. Following synthesis of acyclovir monophosphate (acyclo-GMP) in HSV-tk containing cells, normal cellular enzymes catalyze the sequential synthesis of acyclo-GDP and acyclo-GTP. Acyclo-GTP functions in two ways: (1) as an inhibitor of DNA polymerase and (2) upon incorporation into DNA, acyclovir causes chain termination.

There is a 300-3000 fold difference in the toxicity of acyclovir for HSV-tk containing cells as compared to normal mammalian cells, and acyclovir is noted for its low human toxicity. Acyclovir is available in capsules, ointment, and as a powder for intravenous use. IV administrations of acyclovir

range up to 750 to 3000 mg/m^2/day, although usual IV doses are 5 mg/kg yielding peak plasma levels of 10#g/ml. The $T_{1/2}$ is 2.5 hours in patients with normal renal function. Bioavailability of the oral administration is about 20% (Gilman, et al. (1985) Goodman and Gilman's The Pharmacological Basis of Therapeutics 7th ed.) when compared to IV administration. Related nucleotide analogs, such as E5-(2-bromovinyl)-2' deoxyuridine (BVDU), 9-[4-hydroxy-3-(hytroxymethyl)but-1-yl]guanine, 2'-fluoro-5-iodo-ara-C (F1AC) (Harnden, et al., J. Med. Chem. (1987) 30:1636-1642; Harada, et al. J. Med. Chem. (1987) 30:226-229; DeClercq, et al. Infectious Diseases (1981) 143:846-852) that function similarly are well documented and are available as pharmaceuticals.

An advantage of this type of system is that when the genome comprising the HSV-tk gene is incorporated into a target cell, it will direct the synthesis of HSV-tk, regardless of whether the target cell is an HIV infected T4 lymphocyte, or a lung cancer cell, a pancreatic cancer cell, or any other cell to which the packaging mechanisms can specifically direct the virus. Upon infection of the target cell the Herpesvirus thymidine kinase is produced intra-cellularly. It is only upon application of acyclovir that cell killing occurs.

Cancer chemotherapy has traditionally been directed at inhibition of DNA synthesis. The use of the HSV-tk gene and acyclovir, described above, follows this precedent. However, the target cell can also be killed independently of DNA synthesis. Potentially, this active chemotherapy could shorten the duration of therapy. This is particularly desirable and the transient expression of many chromosomes derived from paporaviruses. As an example, a ribosomal RNA specific ribonuclease, such as angiogenin, or Diphtheria toxin that ADP

ribosylates elongation factor 2 (EF-2) required for protein synthesis. Toxins, together with a provirus or minichromosome containing the mouse mammary tumor (MoMTV) virus 5'LTR which is inducible with dexamethasone, may be used. This type of construct is illustrated in Figure 9.

To protect the packaging cell from angiogenin, a human placental ribonuclease inhibitor (Shapiro and Vallee Pro. Nat. Acad. Sci. (1987) 84:2238-2241) can be cloned into a packaging cell which lacks a glucocorticoid receptor. In a retrovirus construct, the ribonuclease inhibitor can be cloned so as to replace the defective env gene in pCRIPenv-. This would have the effect of protecting the packaging cell from the activity of the angiogenin contained in the retrovirus genome to be packaged. The angiogenin gene can then be cloned downstream of the dexamethasone inducible promoter enhancer derived from the U3 region of the MMTV LTR (Overhauser and Fan J. Virology (1985) 54:133144; Ponta, et al., Proc. Nat. Acad. Sci (1985) 82:1020-1024) or the moloney murine sarcoma virus (MoMSV) enhancer located within MoMSV LTR (Miksicek, et al., Cell (1986) 46:283-290).

The effect of these constructions would be to provide a genome which is targeted to a specific glucocorticoid receptor-containing cell type, using any of the targeting methods described above. When the patient is subsequently treated with a glucocorticoid such as dexamethasone, the target cell synthesizes angiogenin within the cytoplasm of the cell, thereby actively killing the cell.

The actual kill gene, the gene encoding angiogenin, could be replaced with another ribonuclease gene such as that encoding pancreatic ribonuclease, so long as sufficient levels of the ribonuclease inhibitor are present in the packaging cell. Due to the glucocorticoid induction of the ribonuclease, conceivably the packaging cell would

not express the ribonuclease in deleterious quantities. Other ribonucleases and their inhibitors could also be used, for example, pancreatic ribonuclease and various of the natural cytoplasmic ribonuclease inhibitors (Blackburn and Moore, 1982).

Clearly, the concept of ribonucleases and ribonuclease inhibitors can be altered to include proteases and protease inhibitors as well as DNAses and DNAse inhibitors (for example, restriction enzymes and specific methylases the ricin A chain, the diphtheria toxin A chain and the poliovirus AA2 protein). In the case of serine proteases, trypsin could be inhibited by cloning bovine pancreatic trypsin inhibitor into the packaging cell construct (Gebhard, et al. (1986) Proteinase Inhibitors, Elsevier Science Publishers). Alternatively, bdellins could be used to inhibit trypsin, plasmin and sperm acrosin, whereas eglins could be used to inhibit chymotrypsin and subtilisin (Seemuller et al., Proteinase Inhibitors (1986) Elsevier Science Publishers). Other serine proteinase inhibitors include soybean trypsin inhibitor of the Kunitz and Bowman-Birk types (Ikenaka and Norioka Proteinase Inhibitors (1986) Elsevier Science Publishers). Anti-sense RNA can also serve as a cell protecting or inhibiting gene.

While safeguards have been described for use of the subject invention, as exemplified by the use of exogenous induction systems for expression of the kill gene or initiation of a kill cascade in the target cell these protections are not necessary to practice of the subject invention, and may be omitted, depending upon the circumstances of use.

The gene therapy delivery vehicles described herein represent an ideal vector for programming a target cell to produce anti-sense RNA. Anti-sense RNA can be directed at the human oncogenies such as the human H-ras, K-ras, or N-ras genes. The

sequences of the K-ras gene from a human lung cancer cell, and an oncogeny homologous to v-raf from primary stomach cancer have been identified (Shimizu, et al., (1983) Nature 304:497-500; Shimizu, et al. (1985) Proc Natl Acad Sci 82:5641-5634).

For use in humans, particularly for treatment of cancer, pseudovirion yields of 1 x 10^4/cell can be expected. If 2 x 10^6/cell are assumed per ml of culture fluid (Vero, microcarrier fermentation), yields will be nominally 2 x 10^{10}/ml. A career patient in complete remission has a residual 10^9-10^{10}, nominally 5 x 10^9, whole body tumor cell load. Assuming a 200-fold excess of pseudovirions to whole body tumor cell load is desired in treatment, 1 x 10^{12} virions are required/injection (60 ug). This represents the yield from 50 ml of culture. If a treatment involves 25 injections, a single patient treatment requires about 1.25L of tissue culture. The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the invention unless so stated.

EXPERIMENTAL

Basic DNA techniques are as described by Davis (Davis, et al. (1980) Advanced Bacterial Genetics - A Manual for Genetic Engineering). Enzymes for DNA cutting, splicing and ligations are from New England Biolabs (Beverly, MA), and are used according to the manufacturer's specifications. DNA fragments are synthesized on an Applied Biosystems Model 380A synthesizer (Foster City, CA). Conversion of one SH or NH2 construction to another is done by mutagenesis with a synthesized oligonucleotide containing the desired change hybridized to single-strand RF1 M13 mp11 DNA and synthesized to a complete double-stranded circle by Klenow fragment DNA polymerase I (Messing (1983) Methods Enzymology 101:20-78).

Alternatively, complementary DNA strands are synthesized and hybridized to produce a double-strand DNA fragment that can be substituted between two of the unique restriction fragments within the 1.6 kb NdeI-NsiI fragment shown in figure 3 (Henderson, et al. (1986) Clinical Chem 32:1637-1641). Unless otherwise specified, the numbering system refers to MoMuLV starting from the 5'end. DNA transfection is by the method of Graham and Van der Eb (Graham and Van der Eb (1973) Virology 52:456-467)..

Example I

Preparation of pNC1

Packaging Cells

The cells used to test various mutations are NIH/3T3 containing pCRIP env- as described (Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464). pCRIPenv-, shown in figure 1, is a pBR322 amp resistant plasmid containing an engineered MoMuLV. The MoMuLV contains the 5'LTR, is Ψ- with a deletion between BalI (212) and PstI (563) where the numbering is from the 5' end of MoMuLV. The SfiI to NsiI fragment (5382-7054) has been replaced with the equivalent fragment containing a frame-shift mutation at position 6438 within env created by insertion of a ten base linker containing an EcoRI site (Colicelli, et al. (1985) Mol. Gen. Genet. 199:537-539). These packaging cells will be called ΨCRIPenv-cells.

Model Provirus Construction

Plasmid pMOV-Ψ+ (Mann, et al. (1983) Cell 33:153-159) is cut with XhoI (1560) and SfiI (5382). At the unique XhoI site (1560) XhoI-BamI adapters is added, and at the SfiI site (5382) BamHI-SfiI adapters are added:

XhoI-BamHI=TCGAGGAG
CCTCCTAG

BamHI-SfiI=CATCCGGCCNNN

GCCGGN

The BamHI fragment of HSV-tk (3.5 kb) from pxl (Joyner, et al. (1982) Proc. Natl. Acad. Sci. USA 79:1573-1577) is cloned in. This yields a Ψ+ MoMuLV-HSV-tk+ mutant that is packaged to high titer (Bender, et al. (1987) J. Virol. 61:1639-1646), is gag-, -pol-, env+, and is about 350 bp shorter than wild-type MoMuLV. This plasmid is called pNC1 (NovaCell). The structure of the MoMuLV provirus in pNC1 is shown in Figure 2.

Example II

Mutagenesis of env Gene

The unique Nde1-Nsi1 fragment (5401-7054) is subcloned for mutagenesis. Following mutagenesis, the Nde1 fragment is replaced back into pNC1 and transfected into CRIPenv-cells. The resultant virus particles are tested for desirable properties, including infectivity, host-range, host-range alteration by covalent attachment of specific receptors, and cell killing in response to acyclovir by virtue of the expressed HSV-tk. If the env gene is not spliced properly in this construct (expression of both HSV-tk and env is not achieved), other constructions of the two genes, including a separate promotor for env, are made until the two genes are expressed properly. Tests for virus production are by the XC overlay assay, and the reverse transcriptase activity assay (Rowe, et al. (1970) Virology 42:1136-1139).

Preparation of pNC-SH and NH_2 Series

Mutagenesis of Nde1 (7054) is performed as follows: pUC19 contains a single Nde1 site (183 within the pUC19 numbering system) and no Nsi1 sites. The Nde1 site is removed by cutting pUC19 with Nde1, treating with S1 nuclease and religation. The multiple cloning site in pUC19 is cut with EcoRI and

HindIII, to remove the polylinker. In its place is inserted a Nde1-Nsi1 adapter:

TATGAGATGCA
ACTCT

resulting in pUC19a. Insertion of Nde1 (5401)-Nsi1 (7054) into pUC19a yields pUC19-env-, a pUC19 derivative with unique restriction sites within the Nde1-Nsi1 insert, is shown in Figure 3.

The env termination is at 7772. In the region of env 5876-7054, there are codons for 15 cysteine residues. To test whether these cysteine residues are available for coupling with maleimide adducts, as well as the arbitrary mutations creating -SH groups, coupling is made in a manner analogous to that of Henderson, et al. (Henderson, et al. (1986) Clinical Chem 32:1637-1641). The mutated genes are removed from the pUC19 derivatives and replaced into pNC1. A series of variously positioned -SH group in the env gene are constructed as shown in Figure 4. The resulting plasmids are called pNC-SH-1, pNC-SH-2, etc. These plasmids in turn are transfected into CRIPenv- cells creating NC-SH-1, NC-SH-2, etc., cells, producing in turn envSH-1, envSH-2, etc., helper-free virus containing the HSV-tk gene. Analogous constructions which introduce lysine residues with NH-2 groups for coupling are created in pUC19-env-, transferred to pNC1 where they are called pNC-NH-1, pNC-NH-2, etc. These plasmids are transferred into CRIPenv-cells, creating NC-NH-1, NC-NH-2 cells, etc., which in turn will produce envNH-1, envNH-2 helper-free virus particles containing the HSV-tk gene. The packaging cells are designed to produce murine ecotropic viruses and are incapable of infecting human cells.

Example III

Coupling of Specific Antibodies to Native or Modified env Glycoprotein

Tumor-specific antibodies are attached specifically to the protruding env glycoprotein, env-SH or env-NH_2 group, so as to infect only tumor antigen presenting cells. To obtain a modified env glycoprotein, a mutation in the env gene is made yielding a sulfhydryl or amino group free for coupling by organic chemical means. F(ab)'2 fragments of specific antibodies such as an antibody to pancreatic cancer or other tumor-specific antigens or hormone receptors is made to produce cell-specific reagents. Depending upon the reactive moiety available for coupling, for example, amino, carbohydrate, sulfhydryl, these cell-specific reagents are coupled by glutaraldehyde, periodate, or maleimide compounds to the virus gene delivery vehicle comprising the native or modified env glycoprotein. Preferred reagents include maleimide reagents to couple with the sulfhydryl groups of the hinge region of the antibody. See, for example, Ishikawa, et al. (1983) J. Immunoassay 4:209. Examples of other ligands which can be coupled by this method include the antibody to HIV gp120 to target HIV infected lymphocytes, soluble CD4 protein to target HIV infected cells, transferrin to target transferrin receptors, and other members of specific binding pairs such as gastrin, secretin, cholecystokinin or somatomedin C to target their respective cell surface receptors.

Example IV

Coupling of CD4 Protein to Modified env Glycoprotein

A virus designed to infect HIV-infected T4 lymphocytes is also made by fusing the amino acid sequence of the CD4 protein that is recognized by the HIV gp120 to the N-terminus of the env gene. In this way it is possible to direct infection by the HSV-tk containing provirus to T4 lymphocytes specifically elaborating the HIV gp120 on the cell surface. Chandhery, et al. (1988) Nature 355:369-372.

Preparation of pNC-CD4

CD4 is a polypeptide of 55 kDa, about 435 amino acids of which are expressed predominantly on the surface of helper T cells. CD4 is a member of the immunoglobin superfamily and appears to be capable of regulating the proliferation of the CD4 subset of T cells. The N-terminal 178 amino acids of CD4 fused to amino acids 1-3 and 256-613 of Pseudomonas exotoxin A, shown to specifically kill HIV infected cells (Chaudhery, et al. (1988) Nature 355:369-372) is replaced into pNC1 and transfected into CRIPenv-- cells. Virus is tested for infectivity on HIV infected T4 lymphocytes. Acyclovir is then tested for its ability to kill the HIV infected cells.

The first 178 amino acids of CD4 from pVC403 (Chaudhery, et al. (1988) Nature 335:369-372) are placed into the BstE11, Nco1, BstXI, BamHI and TTHIII-I in pUC19-env-. pVC403 are partially cut with Nde1 and Sac1 removing the 178AA of CD4 and part of the Pseudomonas exotoxin A. To insert this fragment into the unique BstE11 site of pUC19-env-, requires an adapter to read in frame.

```
    Glu   Val          His   Met   Lys   Lys   Val
    G A G G T A A C A C C T A T G               CD4
env C T C C A T T G T G G A T A C
```

This linker is ligated to the roughly 250 bp Nde1-Sac1 fragment of pVC403 and then ligated to BstE11 cleaved pUC19-env-. This construct is cut with Nco1 which cuts MoMuLV env at nucleotide 6025, thereby removing the linker from the distal region of env. A Nde1 partial cut removes the approximately 75 bp of domain II of Pseudomonas exotoxin A carried in pVC403. This linear pUC19-env- is then placed back in frame with the following Nde1-Nco1 adapter.

```
          His              His
CD4       C A T A T C A A T C A T G
          G T A T A G T T A G T A C          env
```

This plasmid is cut with Nde1 and Nsi1 and the fragment replaced into pNC1 creating the desired pNC-CD4 with the provirus structure shown in Figure 5.

Example V

Construction of Packaging Cells and Virus Production--Specific for Tumor Cells

Construction of Provirus pNC-HSV-tk(2)

Once mutant viruses are identified as useful (i.e., capable of cell targeted interactions), production binary vector system of Ψ+ provirus and packaging cells are prepared. Experimentally it is convenient to mutate the env gene in a proviral format so as to avoid selecting env+ cells prior to proviral transfection. However, in production, the env gene of choice is transferred to the chromosome of the packaging cell.

To integrate the env gene into the DNA of the packaging cell, the desired Nde1-Nsi1 fragment from the pNC-SH series the pNC-NH series or pNC-CD4 described above is transferred to NIH/3T3 cells. (Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464). Nonsense mutations, or deletions in pCRIPgag-2 mutations are inserted not only at the Xho1 site (1560), but also at Nru1 (2120), Bcl1 (2740), and Sal1 (3705) sites. NIH/3T3 cells are sequentially transformed with pCRIPenv- that has had the EcoRI-Cla1 fragment of env removed, and with the disabled pCRIPgag2.

To transfer the gag, pol, and mutant env genes into a cell destined to become a fully competent packaging cell, three different constructions following a similar format are used. The generalized format is a genome structure of 5'LTR, Ψ-, trans virus gene product driven by the 5'LTR, a second copy of the same trans virus gene product driven by the 5'LTR splice donor and env splice acceptor, and the SV40 poly A.

a) 5'LTR, Ψ-, gag, gag, SV40 poly A.
b) 5'LTR, Ψ, pol, pol, SV40 poly A.
c) 5'LTR, Ψ-, env, env, SV40 poly A.

The structure of pNCgag,gag is shown in Figure 6a. The starting materials for this construct are pCRIPenv- and pMOV-Ψ- (mana et al Cell (1983) 33:153-159). pCRIPenv- is cut with BClI (2740) downstream from the gag terminator (2235). The NdeI (5401) to SbaI (5766) containing the env splice acceptor site is cloned into the BclI site.

Starting with the SbaI site, the two strands of DNA are MoMuLV bp 615-739. At the 5'end (615) is a dsDNA linker of up to about 15 bp to match the PstI (739) of the gag gene. A PstI(739)-BClI(2740) fragment from pMOV-Ψ- is then added. A dsDNA linker of up to about 10 bp designed to join BclI and ClaI adds the SV40 poly A to pCRIPenv-.

The structure of pNCpol,pol is shown Figure 6b. The starting materials again are pCRIPenv- and pMOV-Ψ-. pCRIPenv- is cut with HindIII and pMOV-Ψ- is cut with NruI (2120) and HpaI (5816). The HindIII and NruI sites are joined by a dsDNA adapter of up to about 15 bp. The HpaI site is joined by another dsDNA adapter of up to about 15 bp to the NdeI (5401) of the env splice acceptor fragment, NdeI(5401)-XbaI(5766), from pMOV-Ψ-. A dsDNA adapter, not to exceed 15 bp, joins the SbaI (5766) to the NruI(2120)-HpaI(5816) of the pol gene, again from pMOV-Ψ-. A dsDNA HpaI-ClaI adapter of up to about 15 bp joins this construct into pCRIPenv-. None of the adapters in this construct need maintain a correct reading frame.

The structure of pNCenv,env is shown in Figure 6c. The env gene comes from the desired pNC-env-series: the pNC-SH-series, the pNC-NH-series, or the pNC-CD4-series, or analogous pNC-env-series constructs designed to change the cell-specific host-range of the virus. The backbone of

pNC-env-env is pCRIPgag-2 (Danos and Mulligan, 1988, supra.) pCRIPenv- is cut with HindIII and a dsDNA adapter used to join the NdeI(5401-BSSH II(8212) to the SfiI(5382)-NsiI(7054) env fragment which also contains the env splice acceptor site. The NsiI(7054) site of the altered env gene from the desired pNC-env series is joined in reading frame to the NsiI(7054) of the env gene of pCRIPgag-2.

These three plasmids, pNCgag,gag, pNCpol,pol, and pNCenv,env, are transfected into NIH/3T3 cells in a sequential manner, producing as a result the desired production packaging cell.

With the above-described packaging cells, the second part of the binary vector system is the Ψ+ provirus designed to transfer the desired genes of intent. The structure of pNC-HSV-tk(2) is shown in Figure 7.

Example VI

Construction of Packaging Cell and Provirus for Production of Anti-sense RNA in a Specific Cell Type

Anti-sense RNA can also serve as a cell inhibiting, or cell activity inhibiting gene. The gene therapy delivery vehicles described herein represent ideal vectors for programming a target cell to produce anti-sense RNA. As a specific example, an anti-sense construct for HIV infected T4 lymphocytes is shown. Goodchild, et al. (Goodchild, et al. (1988) Proc Natl Acad Sci, 85:5507-5511) have synthesized anti-sense RNA of 20 bp length to various functional sites of the HIV genome. Oligomers complementary to sites within or near the sequences repeated at the ends of the retrovirus RNA (R region), to splice acceptor, and splice donor sites were found by Goodchild et al. to be most effective.

Construction of pNC-tk-AS

The following four oligonucleotides are synthesized on a DNA synthesizer and inserted into the first BamHI-BamHI insert of HSV-tk in pNC-HSV-tk(2).

Functional Site	BamHI Oligomer 5'-3'	Provirus
Cap	GATCCAGTCAGTCAGTCAGTCAGT GTCAGTCAGTCAGTCAGTCACTAG	NC-HSV-tk-AS$_1$
5' untranslated region	GTACCTGGTCTAACCAGAGAGACC GACCAGATTGGTCTCTCTGGCTAG	NC-HSV-tk-AS$_2$
splice acceptor/ art initiator	GTACACTGGCTCCATTTCTTGCTC TGACCGAGGTAAAGAACGAGCTAG	NC-HSV-tk-AS$_3$
Poly (A) signal	GTACGGCAAGTTTTATTGAGGCTT CCGTTCAAAATAACTCCGAACTAG	NC-HSV-tk-AS$_4$

The structure of pNC-HSV-tk-AS (AS = anti-sense) proviral genome(s) containing the HSV-tk gene and an anti-sense transcript is shown in Figure 8.

Example VII

Construction of an SV40 Binary Vector

The genes encoding polypeptides required for maturation of new SV40 viral particles are VP1, VP2, and VP3 (see Figure 10). VP1, VP2, and VP3 are independently placed on high expression vectors. The constructs are prepared using the MuLV 5'LTR promoter, or the metallothionein promoter or any other strong promoter constructs in the host cell could be used. The structures of the three constructs for VP1, VP2 and VP3 are shown in Figures 11a, 11b, and 11c, respectively. These three constructs are based on pCRIPenv- and SV40, and are designated pNCVP1,VP1, pNCPV2,VP2, and pNCVP3,VP3.

The structure of pNCVP1,VP1 is shown in figure 9a. The numbering system is from MoMuLV unless otherwise indicated. PCRIPenv- is cut from HindIII (212) and ClaI (7674) and SV40 is cut with RsaI (SV40, 1467) and BclI (SV40, 2770). The HindIII and RsaI sites are joined by a dsDNA adapter of up to about 15 bp.

The BClI site is joined to the env splice acceptor (S.A.) site, NdeI-XbaI(5401-5766), with a BclI-NdeI adapter. A second copy of VP1 is ligated into the XbaI site with a XbaI-RsaI adapter. Finally, a BclI-ClaI adapter finishes the construct with the SV40 polyA sequence from pCRIPenv-. None of the adapters in this construct need maintain a correct reading frame.

Figure 11b shows the structure of pNCVP2,VP2. This construct is similar in using pCRIPenv- and SV40. pCRIPenv- is cut with HindIIIand ClaI. The large plasmid backbone is reconstructed with two copies of the SV40 VP2. The small fragment is further cut with NdeI and SbaI to give the env splice acceptor (5401-5766). The HindIII site is joined to the HpaII site of VP2 with an adapter. The VP2 AccI (SV40, 1628) is joined to the S.A. with an adapter to NdeI. The S.A. XbaI is joined with an adapter to a second copy of VP2 at the HpaII (SV40,344). The AccI (SV40,1628) is finally joined back to pCRIPenv- with an adapter to ClaI (5674). None of the adapters in this construct need maintain a correct reading frame.

Figure 11c shows the structure of pNCVP3,VP3. This construct is derived from pCRIPenv- and SV40. pCRIPenv- is cut with HindIII and ClaI to yield the plasmid backbone. The small fragment is further cut with NdeI and XbaI (5401-5766) to yield the S.A. The HindIII (212) site is joined to the SV40 HaeII (832) site with an adapter. The SV40 AccI (1628) is joined with a synthesized dsDNA adapter to the 5 S.A. NdeI (5401). The S.A. XbaI (5766) is joined with an adapter to the VP3 HaeII (32). An adapter of

dsDNA is synthesized to join the AccI-ClaI (SV40 1628-7674) to complete the construct. None of these adapters need maintain a reading frame. The three constructs described in figures 9a, 9b, and 9c are sequentially transfected into a permissive line for SV40 such as CV1, BSC-1 or VERO to yield SV40 packaging cells.

Site-specific mutagenesis of the VP1 construct yields surface SH- or NH2- groups for covalent attachment of cell-specific receptors. These receptors define the host range of the mobile element of the SV40 binary vector. These constructs are patterned after Henderson, et al. Clinical Chemistry (1986) 32:1637-1641. The SH series of constructs are known as pNCSV-SH-1, pNCSV-SH-2, etc., or alternatively for lysine substitutions, pNCSV-NH-1, pNCSV-NH-2, etc.

The mobile element of the SV40 derived vector comprises SV40 nt 2533-5243-294 encoding T antigen and ori, which encodes both the early and late promoters. Downstream from the SV40 late promoter is the desired kill gene. SV40 has a narrow range of DNA size that can be packaged, only a maximum of about 2250 nt can be accommodated. This is sufficient space to encode angiogenin (369 nt), (Kurachi et al. Biochemistry (1985) 24: 5494-5499), the 1605 nt A chain of diphtheria toxin (Greenfield et al., Proc. Nat. Acad. Sci. 80:6853-6857), the 741 nt serine protease from human cytotoxic lymphocytes (Trapani et al. Proc. Nat. Acd. Sci. (1988) 85:6294-6928), ahd the like. The constructions are made in E. coli with pBR322 inserted in the unique BamH1 (2533) site where the numbering system begins at the unique Bgl1 site in the ori. This construct is shown in figure 10 as pNCHSVkill. Prior to transfecting the packaging cell described above, the pBR322 backbone is removed by cutting with BamHI and religation.

The different kill genes in these constructs are synthesized as small DNA fragments (about 100 bp), hybridized and ligated together into the appropriate gene (Henderson, et al. (1986), supra). The synthesized gene is ligated into the SV40 vector, pNCHSVkill.

The above-described binary viral vectors may be used for inhibiting growth of a specific target cell, where the targeting of the vector is provided by a modified virion surface glycoprotein. Growth of the target cell is inhibited either directly by the gene product of a kill gene in the viral vector, or by the action of the kill gene product to produce a cell inhibitory product. The vector finds use both in vitro and in vivo.

Example VIII
Construction of SV40 Pseudovirions

SV40 pseudovirions are constructed in COS-7 cells (Guzman, (1981) Cell 23:175-182). The initial constructs are early T antigen replacement vectors containing the Diphtheria toxin A chain (DT-A) gene under control of heavy chain immunoglobin enhancers. Cloning procedures are as described (Davis, (1980) Advanced Bacterial Genetics - A Manual for Genetic Engineering) Enzymes are used according to recommendations by the manufacturers. DNA is synthesized on an Applied Biosystems Model 380A.

pSV40 is prepared by cloning SV40 DNA in pBR322 at the unique EcoR1 site. The PvuII-PstI fragment of pBR322 is replaced with SalI-PstI of pBR327 (Lusky & Botchan Nature (London) (1981) 293:79-81) to remove the poison sequence. A multiple cloning site (MCS), the toxin MCS, is synthesized and cloned into TaqI (SV40 numbering system nt 4739). The two small BbvII fragments (nt 850-2904, and 2904-4416) of T antigen are removed. The small polyA-containing SV40 BclI-BamHl fragment (nt 2770-2533) is duplicated

and a second multiple cloning site, (polyA MCS is inserted in the joint between the duplication, creating pNCSV1 shown in Figure 13.

In pNCSV1, the SV40 ori and late functions VP1, VP2, VP3, agno gene, and late polyA site, are intact. The early region contains: a short region of the 5' coding region of T antigen, a terminator terminate the T antigen transcript, a MCS for the insertion of DT-A and regulatory enhancer and promoter elements (IgG enhancer 289 bp AluI fragment, and heavy chain promoter), the SV40 splice site, the SV40 early polyA site, and a second multiple cloning site, the polyA MCS. The polyA MCS is for an additional AluI enhancer and an anti-sense T3 RNA polymerase promoter (O'Conner, et al. J. Bacteriol (1987) 169:4457-4462; Deuschle, et al. Proc. Nat. Acad. Sci. (1989) 86:5400-5404). This yields 1310 bp of available coding space in the SV40 pseudovirion. SV40 can package minichromosomes 75-102% of the wild-type 5243 bp (Subramani, Anal. Biochem. (1983) 135:1-15).

The gap of 1310 nt in T antigen is replaced with: a translation terminator, an oligonucleotide spacer, and a polyA signal (TGA-oligo(n=20)-AATAA) to terminate the first few nucleotides of T,t antigen, the 279 bp IgG heavy chain enhancer (AluI fragment f pSer.Alu.a) (Hayday, et al. Nature (1984) 307:334-340), the IgG heavy chain promoter, a second 279 bp IgG heavy chain enhancer, and the 582 nt DT-A gene (Maxwell, et al. Cancer Res. (1986) 46:4660-4664). The SV40 early replacement vector is removed from this plasmid by cutting at the single EcoRI site, gel purified, and circularized by ligation. Transfection of DNA by electroporation shows the toxicity and cell specificity of this construct. Transfection of COS-7 cells with this yields pseudovirions. This construct is expected to show an 80-90% specificity for B cells as compared to other cell types.

Example IX

Construction of Anti-Sense SV40 Pseudovirions

SV40 pseudovirions are constructed as described in Example VIII. An anti-sense construct can be added, driven by the T3 RNA polymerase promoter (O'Conner, et al. J. Bacteriol. (1987) 169:4457-4462). T3 RNA polymerase and the T3 promoter are very specific and function in eukaryotic cells (Deuschle, et al. Proc. Nat. Acad. Sci. (1989) 86:5400-5404). Inclusion of the T3 RNA polymerase in COS-7 cells, and the T3 promoter at the 3'end of the DT-A gene yields anti-sense RNA inactivating DT-A mRNA. Anti-sense RNA is not present in the target tumor cells as they do not contain the T3 RNA polymerase. This construct is shown in Figure 14.

SV40 infects most cell types of monkey and human origin in vitro, and in vivo. SV40 efficiently infects human lymphocytes (Oppenheim, et al. Proc. Nat. Acad. Sci. (1986) 83:6925-6929; Karlsson, et al. Proc. Nat. Acad. Sci. (1985) 82:158-162). The SV40 pseudovirions described above will provide early pseudovirion in vitro efficacy data. The SV40 work can be used to obtain preliminary animal model in vivo therapeutic data.

Example X

Construction of LPV Pseudovirions

LPV has a much narrower host range than SV40, infecting only B cell tumor cells of monkey and human origin. The host range restriction is a function of both enhancer and VP1 sequences. LPV enhancers restrict gene expression to B and T cells (Mosthaf, et al. Nature (1985) 315:597-600; Chen, et al. J. Virol. (1989) 63:2204-2214), whereas VP1 restricts LPV to B cells (Kanda, et al. J. Virol. (1986) 59:531-534). Figure 15 shows schematic representations of the SV40 and LPV genomes (Fried, et al. (1986) supra; Pawlita, et al. Virology (1985) 143:196-211). The similarity is striking. The

genome organization is identical. The two viruses do not cross-react, yet show 41% DNA sequence identity. The amino acid sequence of VP1, the major virion polypeptide, shares over 54% identity.

Issues of DT-4 cloning, target cell toxicity, and tissue specific enhancers will be resolved in the SV40 work described above. The LPV work will be designed to produce safe, contained, replication incompetent pseudovirions. Packaging cells will be constructed using COS-1 and COS-7, Psi-2, Psi-AM cells, and two gene separation of virion functions on separate host cell chromosome inserts, as models (Guzman, et al. Cell (1981) 23:175-182; Mann, et al. Cell (1983) 53:153-159; Cone, et al. Proc. Nat. Acad. Sci. (1984) 81:6349-6353; Danos, et al. Proc. Nat. Acad. Sci. (1988) 85:6460-6464).

The goal is to produce the highest possible titer construct. This is achieved by designing an appropriate packaging cell. Packaging cells encode virion proteins to be supplied in trans to pseudovirion production. The highest pseudovirion titer may be achieved with lymphoma packaging cells (BJA-B) (Klein, et al. Proc. Nat. Acad. Sci. (1974) 71:3283-3286) in suspension culture producing LPV capsids. Alternatively an SV40-LPV hybrid capsid produced in CV-1, Vero, or BJA-B packaging cells may be best. The change of ecotropic to amphitropic host range of Moloney murine leukemia virus gene delivery vehicles involved retaining the ecotropic gag and pol genes, but exchanged the env gene from the amphitropic murine leukemia 4070A strain. Similarly, the requirement for an LPV vector is to utilize the LPV VP1. VP2, VP3, ori, and T antigen could well be from SV40. T antigen, VP1, VP2, and VP3 expression in the packaging cells will be cloned into the chromosome of the packaging cells with the amphitropic retrovirus Psi-AM. These genes will be controlled by the inducible metallothionein promoter.

This promoter system has been shown to be compatible with the production of T antigen for SV40 pseudovirion production in CV-1 cells (Settleman, et al. Proc. Nat. Acad. Sci. (1988) 85:9007-9011).

Figure 16 shows pNCLVP2, an LPV ori miniplasmid construct prepared in BJA-B packaging cells containing LPV virion polypeptides. This appears similar to Figure 13, pNCSV1, except the sequences are LPV and the restriction cut sites are different, yet the functional regions are identical. Additionally, a minichromosome with an SV40 ori, SV40 T antigen, and LPV VP1, VP2, and VP3, will be constructed by exchanging the SmaI-NcoI ori fragment of pNCLV2 for the KpnI (nt 294)-EcoN1(nt 5027) ori fragment of pNCLV1.

Appropriate packaging cells will be produced from the results of this work as described above for retroviruses (See Example V). For minichromosome packaging into pseudovirions, the requirements are an ori and a circular genome 75-102% of 5243 bp. Larger plasmids are not packaged. The production minichromosome will contain two copies of the toxin insert shown in Figure 14 oriented in opposite directions as shown in Figure 17. Sequences on either side of the ori to the toxin MCS will be unrelated DNA composed of lac Z sequences to make an appropriate sized (5240 bp) construct.

The above-described binary viral vectors may be used for inhibiting growth of a specific target cell. Growth of the target cell is inhibited either directly by the gene product of the kill gene in the viral vector, or by the action of the kill gene product to produce a cell inhibitory product. The compositions and methods may find use, for example, as vehicles to transiently express foreign genes in HIV-1 infected cells, and other cell types when the viral agent is a papovavirus. As a prototype of the papovaviruses, the advantages of, for example, SV40

as an ablative therapeutic or more generically virus therapy, are two-fold: (1) expression of SV40 minichromosomes as transient genetic engineering of target and non-target cells does not occur, and (2) SV40 is stable and manufacturable. SV40 minichromosomes amplified by T antigen dependent replication as well as nonamplified constructs may find use. Papovaviruses offer the additional advantage that they generally have a narrow host range and those might be used to develop tissue specific virus therapy.

Example XI

Construction of LPV-76

LPV-76 is a mutant of LPV that infects both T cells and B cells of monkey and human origin (Kanda, et al. J. Virol. (1986) 143:196-211). The mutations in LPV-76, as compared to LPV, responsible for increasing the host range to include T cells are three amino acid changes in VP1. Otherwise the viruses are identical. To build on the therapeutic described in Example X requires two additions: First the packaging cell must express the VP1 of LPV-76 instead of the VP1 or LPV. Second the DT-a toxin insert shown in Figure 14, and incorporated in the production minichromosome is Figure 17, must be redesigned to limit gene expression to T cells.

The construction of the packaging cell is as follows. The appropriate mutations in VP1 are removed from LPV-76 on a 3 kb PatI fragment (Kanda, et al. J. Virol. (1985) 55:96-100). This fragment is further reduced to contain only VP1. Packaging cells are produced in CV-1, VERO, or BJA-B cells as described in Example X.

A T cell ablative therapeutic is constructed using DT-A toxin under the expression control of the T cell receptor (TCR) enhancers/promoters. The T cell receptor is a cell surface heterodimer molecule composed of either alpha + beta or gamma + delta

chains. The alpha gene is expressed in the classical helper and killer T cells (CD4+CD8- and CD4-CD8+) whereas the delta chain is expressed primarily in the CD4-Cd8- T cells. A single locus containing both the alpha and delta genes is of interest because t cell derived acute and chronic lymphocytic leukemia (T-ALL and T-CLL) involve translocations of this locus. An alpha chain enhancer within the 0.5 kb J21-0.5 Bgx and J21-0.5 Pv fragment described by Winoto et al (Winoto, A., and D. Baltimore, EMBO J. (1989) 8:729-733), has been sequenced as a minimal 230 bp PvuII/BglII enhancer fragment. This enhancer lacks binding sites for nuclear factors AP1, AP2, AP3, AP4, Spl, NF-A1 (NF-A2) or NF-kB. This enhancer is T cell specific, being expressed in T-lymphoid cells only, not in B-lymphoid for non-lymphoid cells. This enhancer will control DT-A expression as shown in Figure 18.

the toxin insert shown in Figure 18 is inserted in the production plasmid shown in Figure 17 replacing the toxin insert shown therein. Other T cell enhancers which can be used in this construction include the enhancers found for the genes encoding the TCR beta and the CD3 delta subunit (Georgopoulos et al. EMBO J. (1988) 7:2401-2407).

A therapeutic exclusively effective against a subset of T cells, HIV-1 infected T cells, can be constructed. HIV-1 is the causative agent of AIDS and infects CD4+ cells. Unique to HIV-1 infected cells is the enhancer defined by the tat protein encoded by the HIV-1 genome, and TAR, the mRNA sequence responsive to the tat protein and encoded in the 0 to +80 transcription sequence of the HIV-1 5'-LTR.

The packaging cell used for this therapeutic uses the VP-1 from LPV-76 described earlier in this Example. The enhancer/promoter insert is shown in Figure 19.

The HIV 5'-LTR from -350 to +80 is derived as a KpnI-HindIII fragment from pHIV-CAT (Peterlin et al. AIDS (1988) 2 suppl.:529-540). The insert in Figure 19 replaces the toxin insert in the production minichromosome shown in Figure 17. This therapeutic will kill only T cells infected with HIV-1.

Example XII

Constructino of BKV Pseudovirions

BK virus (BKV) is a human papovavirus isolated from the urine of a renal allograft recipient undergoing immunosuppressive therapy. BKV infects humans in childhood and persists in a latent from without causing any serious disease. BKV is routinely isolated from the urine of immunosuppressed patients and pregnant women. Molecular and biochemical studies have shown that BKV resembles SV40. Indeed the relationship between SV40 and LPV; the molecular organization of T antigen, VP1, VP2, and VP3 is identical; BKV and SV40 share 73% amino acid identity. The major difference in sequence occur in the enhancers/promoters of the transcription located near the origin of DNA replication, and the late region (VP1) of the genome. In transgenic mice containing the entire BKV genome, BKV antigens are found exclusively in kidney and liver.

Presumably the tisssue tropisim of BKV for kidney and liver cells is a result of VP1 and the transcriptional enhancers/promoters. To develop a therapeutic for primary hhepatocellular carcinoma a packaging cell containing thhe VP1 of BKV is required. As in Examples X and XI, a new packaging cell expressing the VP1 of BKV is constructed in CV-1, VERO, or BJA-B cells. The ori, T antigen, VP2, and VP3 may be obtained from BKV or SV40 as described in Example X.

To further narrow the expressin of the DT-A toxin to liver cells, particularly liver cancer

cells, the enhancer/promoter controlling the Hepatitis virus B virus surface antigen is used. hepatitis B virus has been cloned and sequenced. The large surface protein gene of Hepatitis B virus is controlled by enhancers/promoters functional only in hepatoma cells (Yee, Science (1989) 246:658-661). This promoter/enhancer is contained within the fragment 966-1402 of the Hepatitis B virus sequence (Chang, et al. Molec Cell Biol. (1989) 9:5189-5197). The BKV Diphtheria A toxin and promoter/enhancer insert is shown in Figure 20. This enhancer/promoter toxin insert replaces the toxin insert in the production minichromosome, Figure 17. Thus DT-A will only be expressed in liver cancer cells.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for preparing an at least substantially replication-defective virion capable of introducing a kill gene into a target cell, said method comprising:

transforming into a packaging cell at least a first and a second construct, wherein said first construct comprises a provirus having the following structure:

5'LTR--P.S.--(V.G.)0-2--G.I.--(V.G.)0-2--3'LTR

wherein said P.S. intends a viral packaging sequence, said V.G. intends one of the genes encoding a trans acting polypeptide required for maturation of new virus particles (trans gene), and said G.I. intends a kill gene; and wherein said second construct, and optionally a third and a fourth construct, has the following structure:

$$(5'LTR)_a-(P)_b-(V.G.)_c-T.R.$$

wherein said P intends a promoter other than 5'LTR, T.R. intends a transcriptional termination region, a, b, and c are integers, a and b being from 0-1, where the sum of a + b is at least 1, and c is 1-3; wherein none of said constructs comprises more than two of said trans genes; and

growing said packaging cell whereby said genes are expressed and said virion is obtained.

2. The method according to Claim 1, wherein said trans genes comprise retrovirus gag, pol and env genes.

3. The method according to Claim 2, wherein one of said trans genes is a modified env gene comprising a nucleic acid sequence encoding one member of a specific binding pair.

4. The method according to Claim 2, wherein said constructs comprise at least two copies of each of said gag, pol and env genes.

5. The method according to Claim 1, further comprising binding nondiffusively to a cell surface glycoprotein of said virion one member of a specific binding pair (sbpm).

6. A binary vector capable of producing a mobile genetic element characterized as infectious, at least substantially replication-defective and capable of introducing at least one copy of a kill gene into a target cell, said binary vector comprising:

a packaging cell comprising at least one copy of each of the genes encoding trans acting polypeptides required for maturation of new virus particles (trans genes); and

a construct comprising a 5'LTR, a viral packaging sequence, at least one copy of said kill gene and a 3'LTR.

7. The binary vector according to Claim 6, wherein said trans genes are retrovirus gag, pol and env genes.

8. The binary vector according to Claim 6, wherein said kill gene is an HSV-tk gene.

9. The binary vector according to Claim 6, wherein said construct comprises at least two copies of said kill gene.

10. A method for altering the host range of a mobile genetic element, said method comprising: transforming into a packaging cell at least a first

and a second construct, wherein said first construct comprises a provirus having the following structure:

5'LTR--P.S.--(V.G.)0-2--G.I.--(V.G.)0-2---3'LTR

wherein said P.S. intends a viral packaging sequence, said V.G. intends one of the genes encoding a trans acting polypeptide required for maturation of new virus particles (trans gene), and said G.I. intends a gene of interest; and wherein said second construct, and optionally a third and a fourth construct, has the following structure:

$$(5'LTR)_a-(P)_b-(V.G.)_c-T.R.$$

wherein said P intends a promoter other than 5'LTR, T.R. intends a transcriptional termination region, a, b, and c are integers, a and b being from 0-1, where the sum of a + b is at least 1, and c is 1-3; wherein none of said constructs comprises more than two of said trans genes;

growing said packaging cell whereby said genes are expressed and said mobile genetic element is obtained, the host range of said mobile genetic element being altered by modifying the cell surface glyco-protein of said mobile genetic element so that it comprises one member of a specific binding pair (sbpm),

said modifying comprising:

a. binding said sbpm nondiffusively to said glycoprotein of said mobile genetic element after said growing; or

b. altering the trans gene encoding said glycoprotein so that a modified glycoprotein comprising a functional moiety for binding is produced; and

binding said sbpm nondiffusively to said functional moiety after said growing; or

c. altering the trans gene encoding said glycoprotein so that a modified glycoprotein comprising said sbpm is produced.

11. The method according to Claim 10, wherein said sbpm is a receptor ligand or an antibody or fragment thereof to a tumor specific antigen or a viral protein.

12. The method according to Claim 4, wherein said sbpm is CD4, an antibody to hepatitis B surface antigen, or fragment thereof capable of binding to the second member of said specific binding pair.

13. A mobile genetic element characterized as derived from a mammalian virus, at least substantially replication-defective and comprising one member of a specific binding pair (sbpm) whereby it is capable of infecting a specific target cell comprising the second member of said specific binding pair.

14. The mobile genetic element according to Claim 13, wherein said mammalian virus is a retrovirus.

15. The mobile genetic element according to Claim 13, wherein said target cell comprises at least two different second members of a specific binding pair.

16. The mobile genetic element according to Claim 13, wherein said second sbpm is an antigen at least substantially specific for colon cancer or stomach cancer.

17. The mobile genetic element according to Claim 13, wherein said second sbpm is gp120 or hepatitis B surface antigen.

18. The mobile genetic element according to Claim 13, wherein said second sbpm is a peptide hormone receptor.

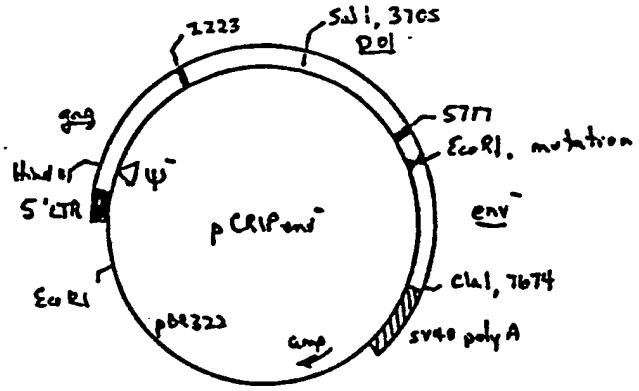

2223
Sal I, 3705
pol
gag
STT
EcoRI, mutation
Hind III
ψ⁻
5' LTR
pCRIPenv⁻
env⁻
Eco RI
Cla I, 7674
pBR322
amp
SV40 poly A

Figure 1

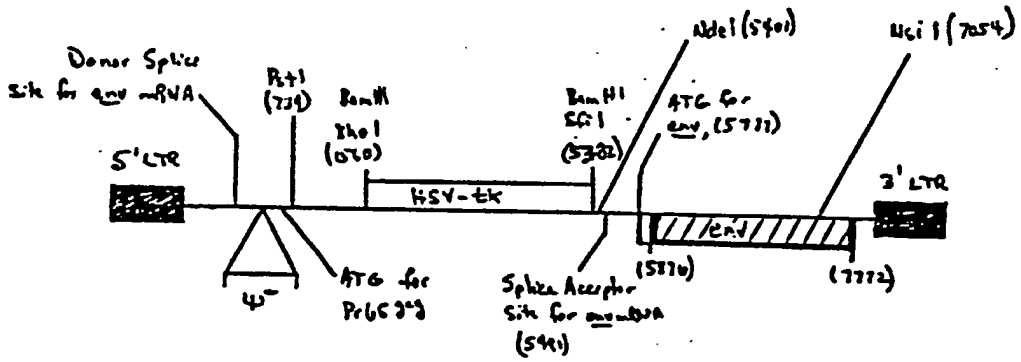

Donor Splice
site for env mRNA
Pst I
(734)
BamH
Xho I
(870)
5' LTR
HSV-tk
BamHI
Sal I
(5332)
NdeI (5401)
ATG for
env, (5771)
NcoI (7054)
3' LTR
env
(5870)
(7772)
ψ⁻
ATG for
Pr65gag
Splice Acceptor
site for env mRNA
(5911)

Figure 2

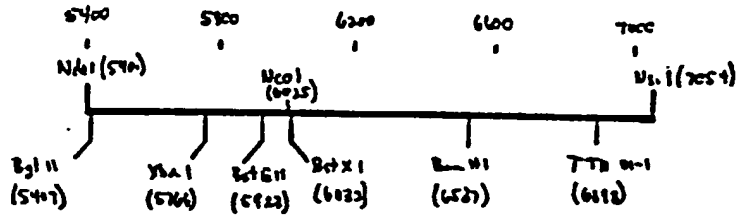

5400
5800
6200
6600
7000
Nde I (5701)
Nco I (6025)
Bgl II (5401)
Xba I (5766)
Bst EII (5923)
Bst X I (6023)
Bam HI (6527)
Hin d III (6919)

Figure 3

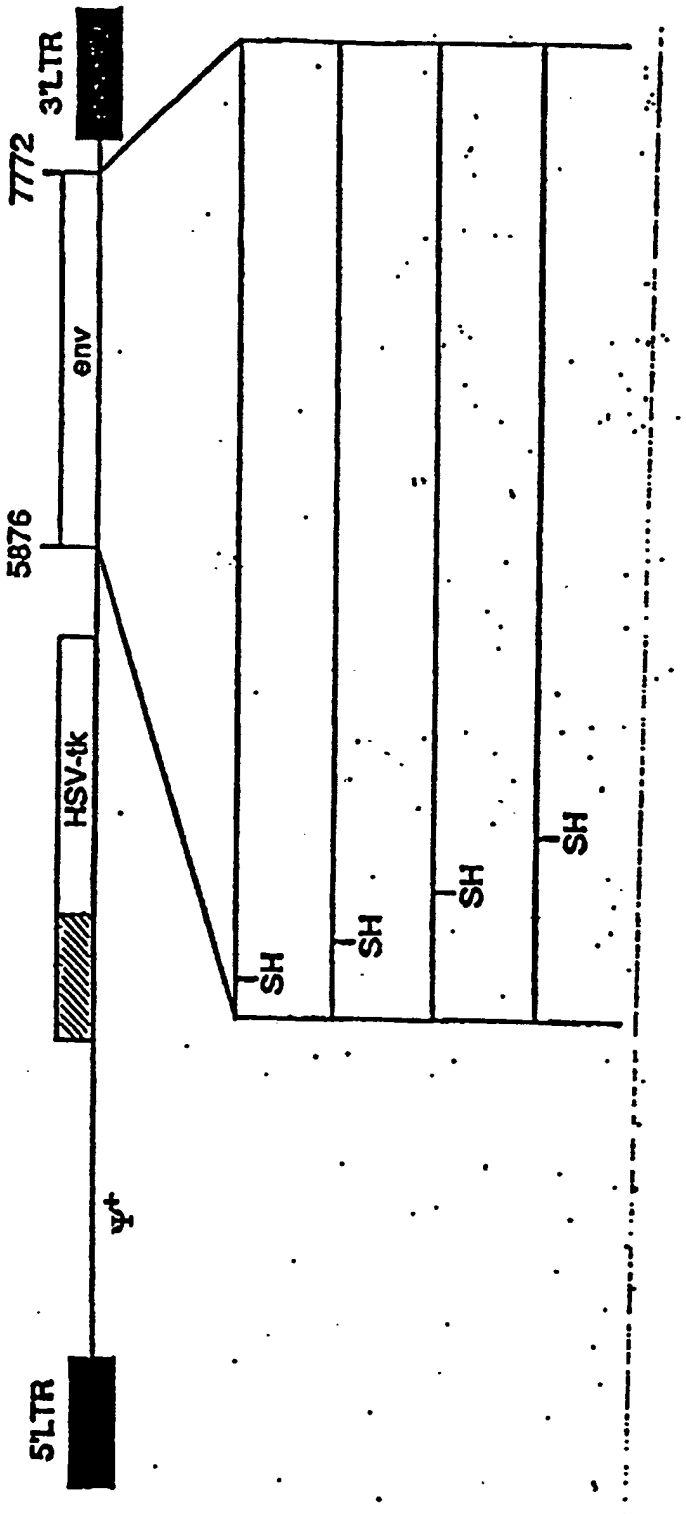


Figure 4

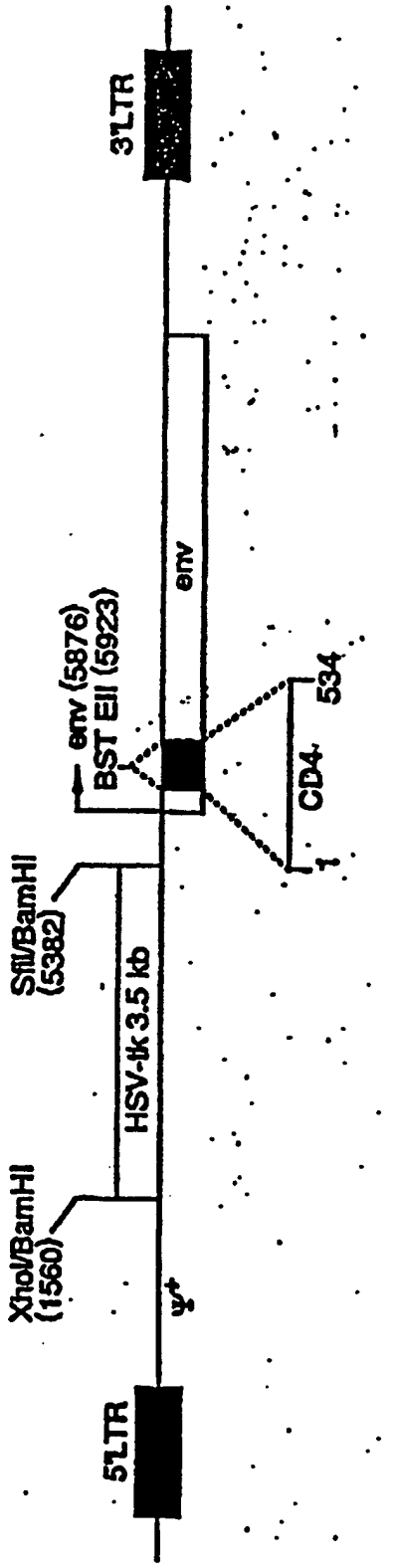

3'LTR
env
env (5876)
BST EII (5923)
534
CD4
1
SfiI/BamHI
(5382)
HSV-tk 3.5 kb
XhoI/BamHI
(1560)
ψ+
5'LTR

Figure 5

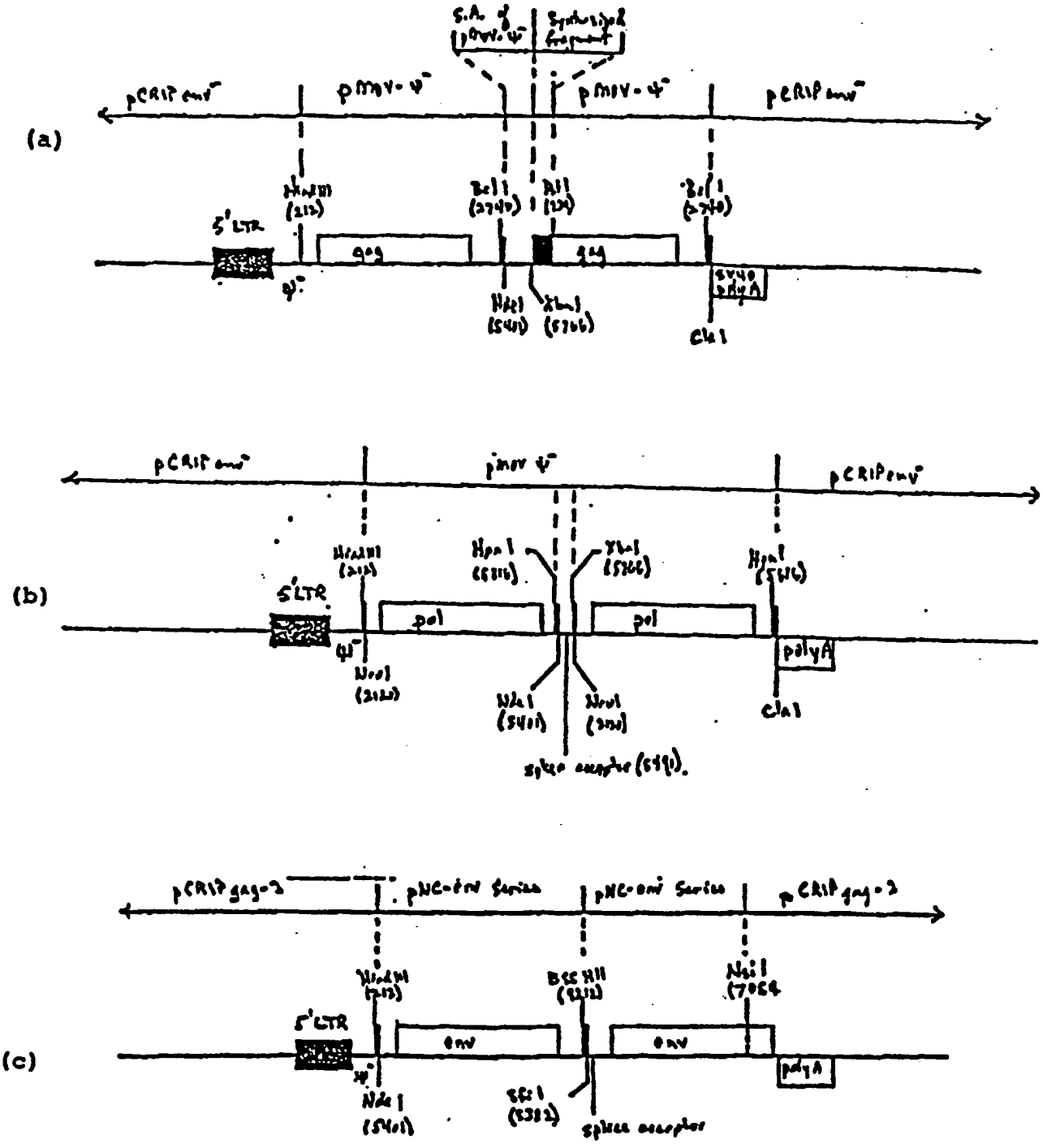

(a)
pCRIPenv⁻
pMOV-Ψ⁻
pMOV-Ψ⁺
pCRIPenv⁻
5'LTR
gag
gag
polyA
Ψ⁻
Cla I
(b)
pCRIPenv⁻
pMOV Ψ⁻
pCRIPenv⁻
5'LTR
pol
pol
polyA
Ψ⁻
Cla I
(c)
pCRIPgag-3
pNC-env Series
pNC-env Series
pCRIPgag-3
5'LTR
env
env
polyA
Ψ⁻
splice acceptor

Figure 6

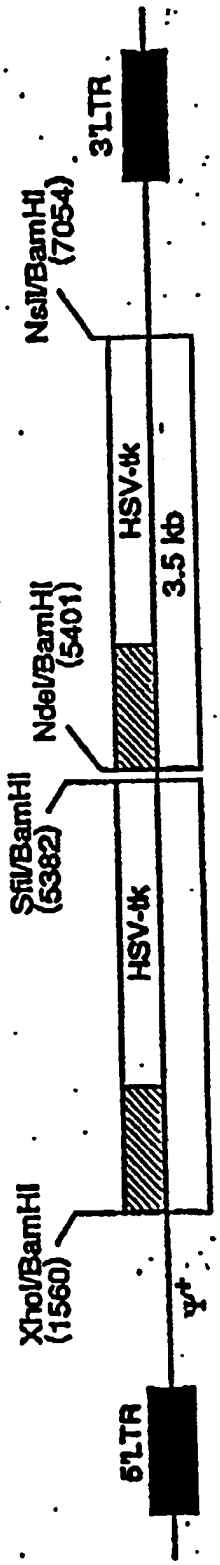

3'LTR
NsiI/BamHI (7054)
HSV-tk
3.5 kb
NdeI/BamHI (5401)
SfiI/BamHI (5382)
HSV-tk
XhoI/BamHI (1560)
ψ+
5'LTR

Figure 7

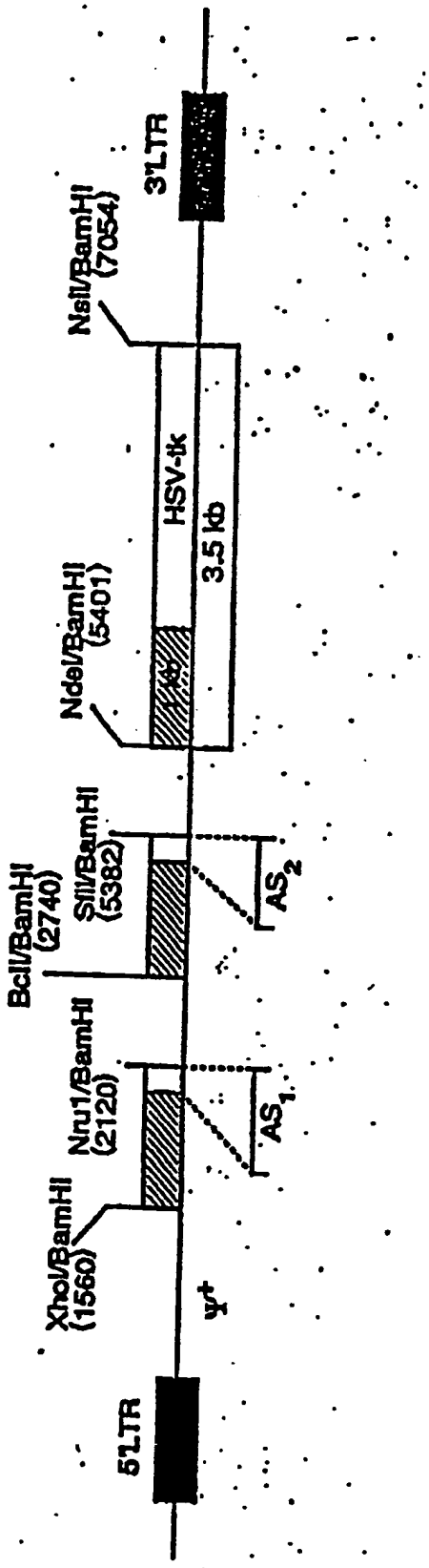

3'LTR
NsiI/BamHI (7054)
HSV-tk
3.5 kb
NdeI/BamHI (5401)
SIII/BamHI (5382)
BclI/BamHI (2740)
AS2
Nru1/BamHI (2120)
AS1
XhoI/BamHI (1560)
ψ+
5'LTR

Figure 8

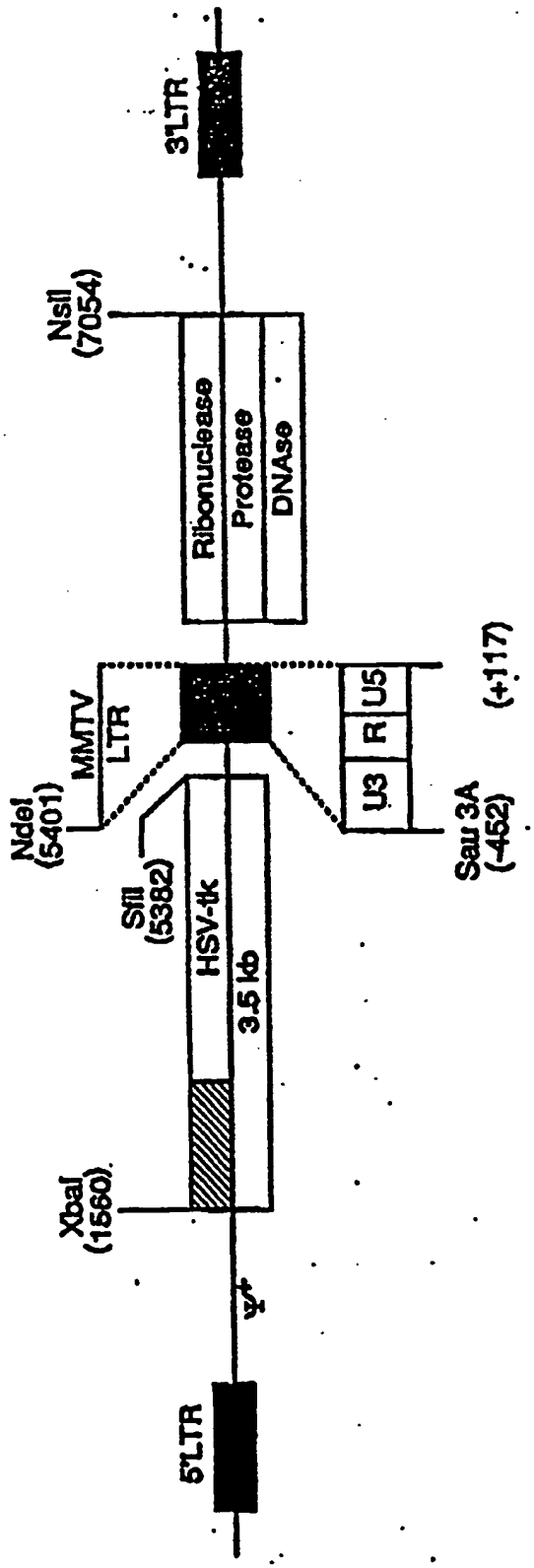

3'LTR
NsiI (7054)
Ribonuclease
Protease
DNAse
MMTV LTR
NdeI (5401)
SfiI (5382)
HSV-tk
3.5 kb
XbaI (1660)
ψ+
5'LTR
U5
R
U3
(+117)
Sau 3A (-452)

Figure 9

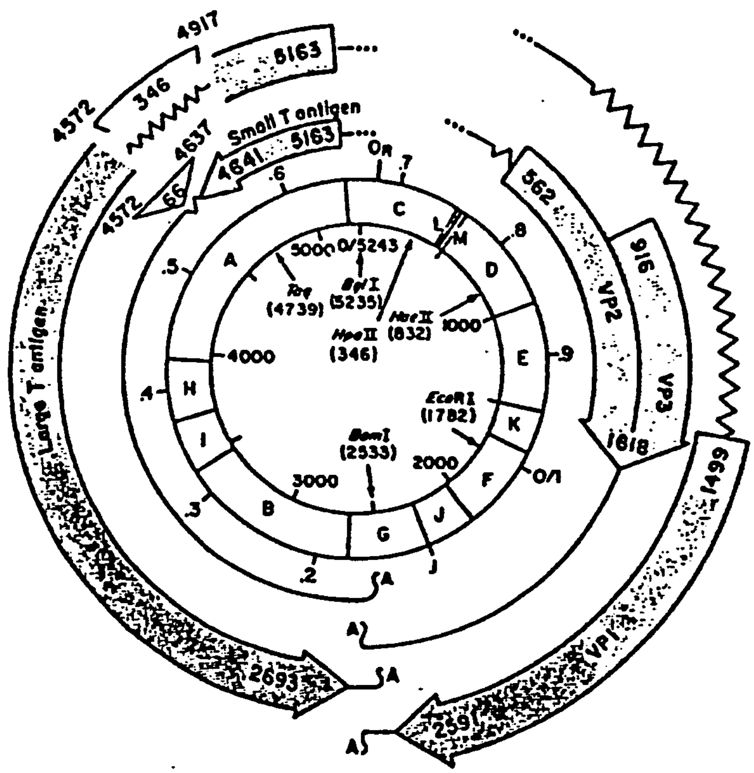

4917
5163
4572
346
Small T antigen
4637
4641
5163
4572
66
Large T antigen
2693
562
VP2
916
VP3
1818
1499
VP1
2591
OR
.7
.6
.8
.5
.9
.4
.3
.2
O/1
C
L
M
D
E
K
F
J
G
B
I
H
A
0/5243
BglI
(5235)
HpaII
(346)
HaeII
(832)
1000
4000
EcoRI
(1782)
BamI
(2533)
2000
3000
(4739)

Figure 10

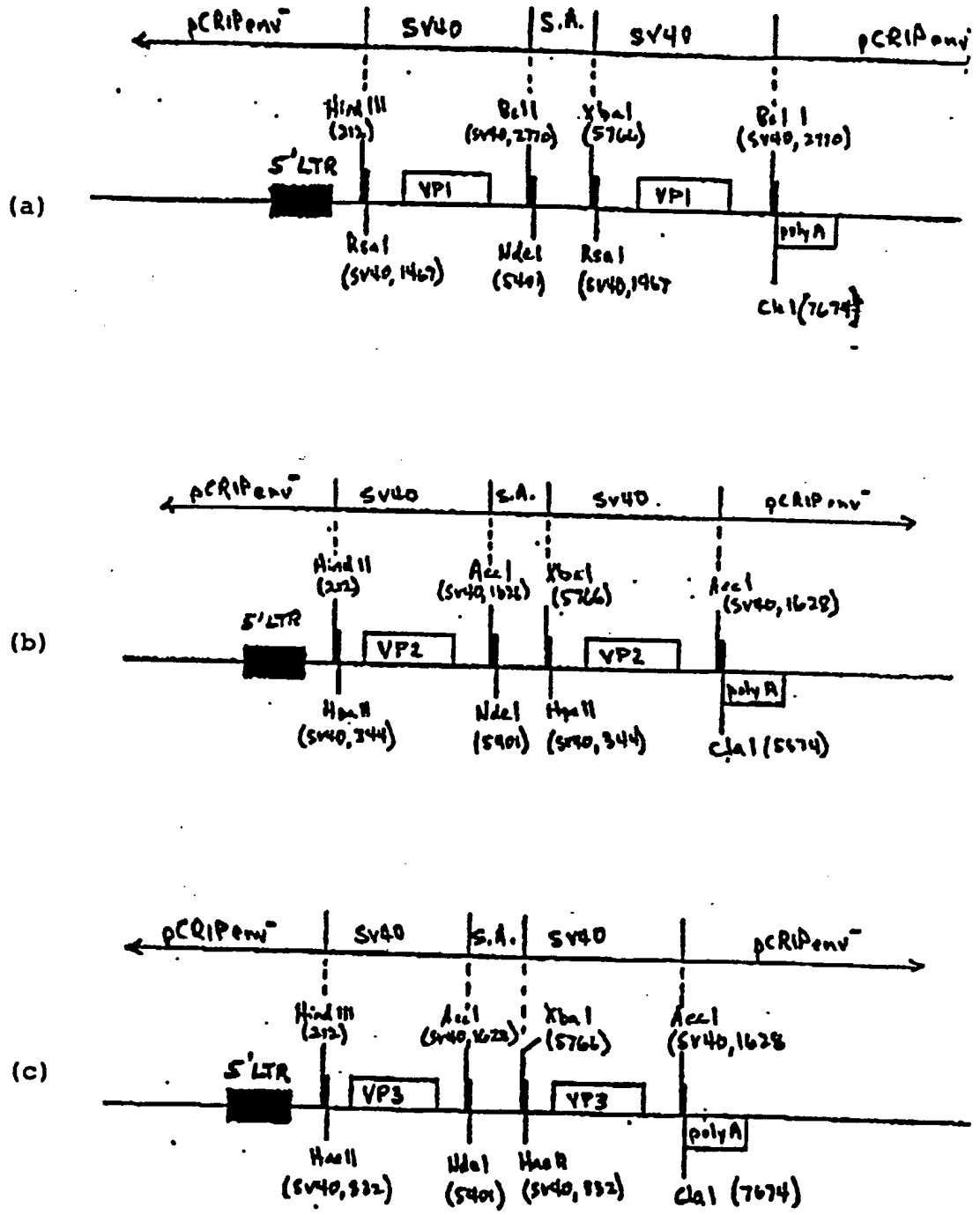

(a)
pCRIPenv⁻
SV40
S.A.
SV40
pCRIPenv⁻
HindIII (212)
BclI (SV40, 2770)
XbaI (5766)
BclI (SV40, 2770)
5'LTR
VP1
VP1
polyA
RsaI (SV40, 1467)
NdeI (5401)
RsaI (SV40, 1967)
ClaI (7674)
(b)
pCRIPenv⁻
SV40
S.A.
SV40
pCRIPenv⁻
HindII (212)
AccI (SV40, 1628)
XbaI (5766)
AccI (SV40, 1628)
5'LTR
VP2
VP2
polyA
HpaII (SV40, 244)
NdeI (5401)
HpaII (SV40, 344)
ClaI (5674)
(c)
pCRIPenv⁻
SV40
S.A.
SV40
pCRIPenv⁻
HindIII (212)
AccI (SV40, 1628)
XbaI (5766)
AccI (SV40, 1628
5'LTR
VP3
VP3
polyA
HaeII (SV40, 332)
NdeI (5401)
HaeII (SV40, 332)
ClaI (7674)

Figure 11

Fig 3

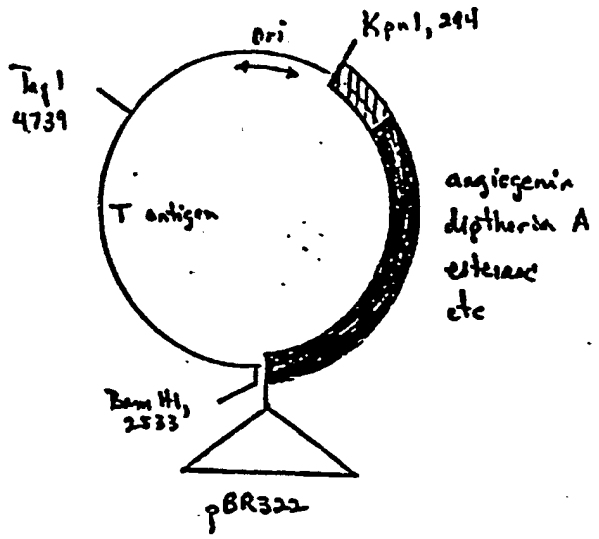


Figure 12

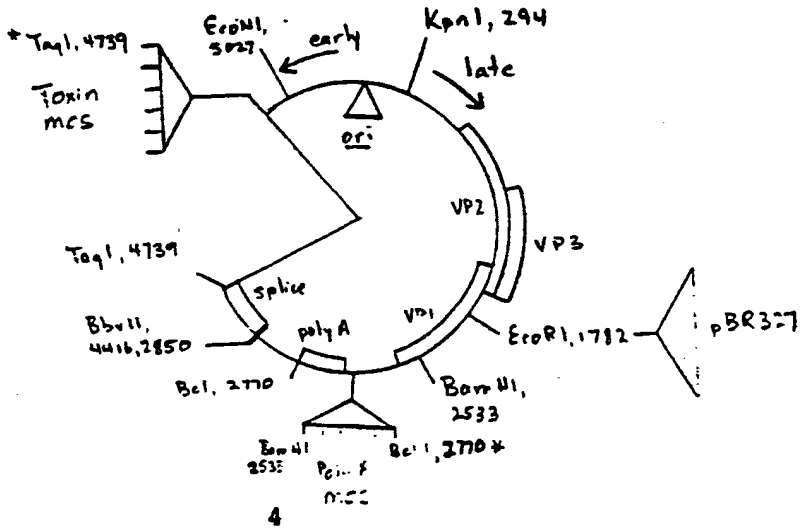

*TaqI, 4739
EcoNI, 3027
early
KpnI, 294
late
Toxin mcs
ori
VP2
VP3
TaqI, 4739
splice
BbvII, 4416, 2850
poly A
VP1
EcoR1, 1782
pBR327
BclI, 2770
BamHI, 2533
BamHI 2533
BclI, 2770*
4

Figure 13

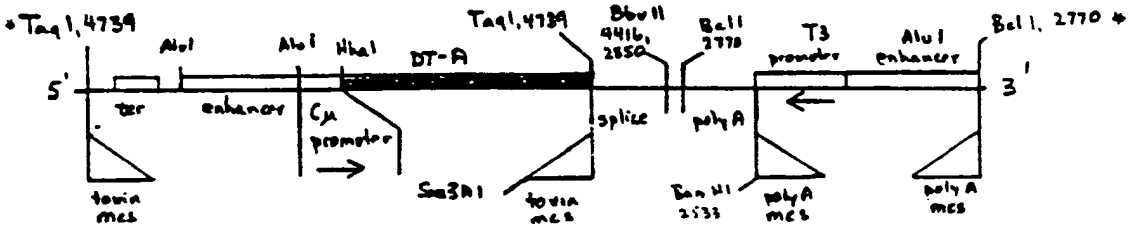

*TaqI, 4739
AluI
AluI
HhaI
DT-A
TaqI, 4739
BbvII 4416, 2850
BclI 2778
T3 promoter
AluI enhancer
BclI, 2770 *
5'
3'
ter
enhancer
Cμ promoter
splice
poly A
Sau3AI
BamHI 2533
poly A mcs
poly A mcs
tevia mcs
tevia mcs

Figure 14

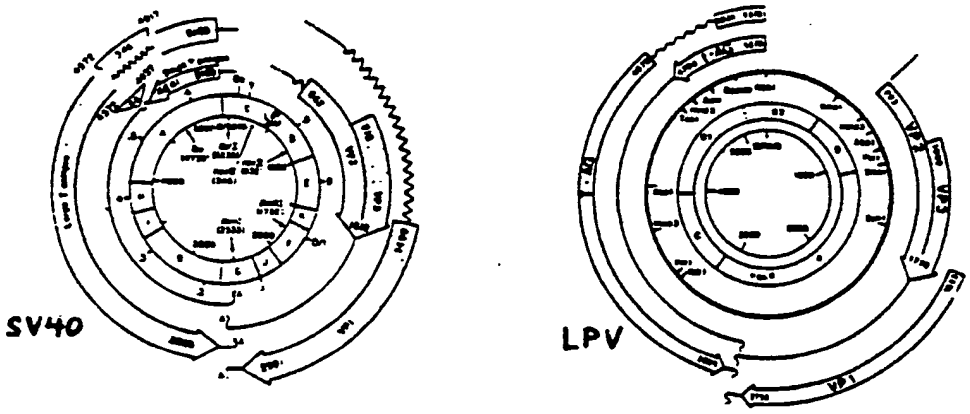


Figure 15

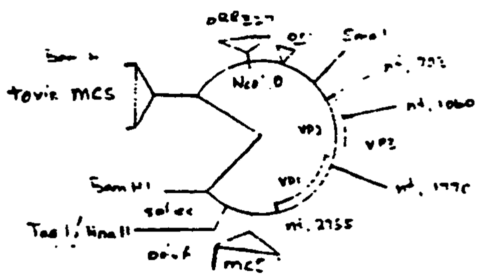
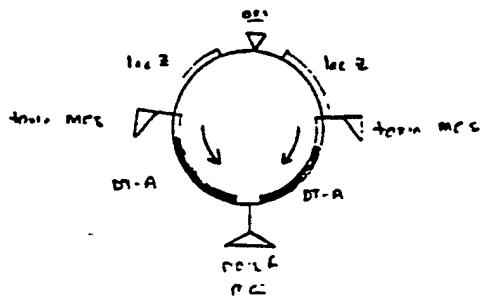


Figure 16



7

Figure 17

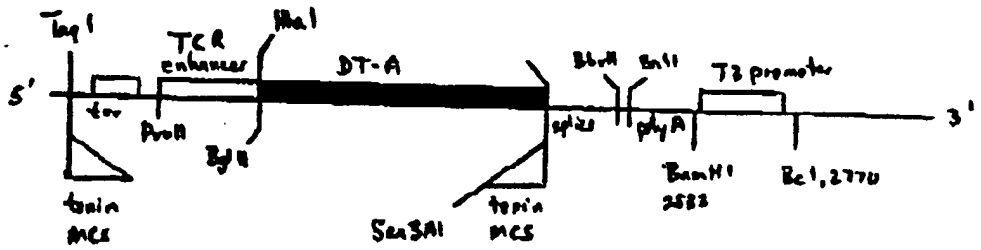

Taq I
TCR enhancer
Hha I
DT-A
BbvII
EcoRI
T3 promoter
5'
3'
tev
PvuII
BglII
splice
polyA
BamHI 2583
EcI,2770
tonin MCS
SmaI
SmaI
topin MCS

Figure 18

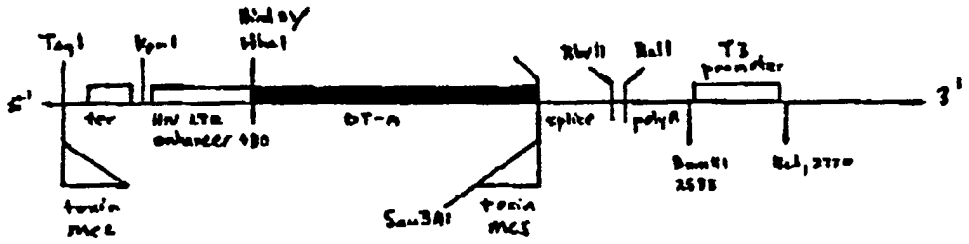
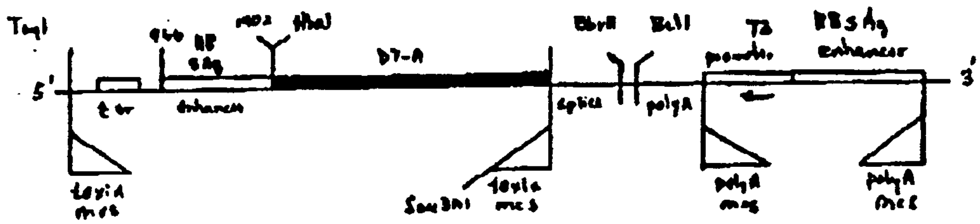

5'
3'
tev
HIV LTR enhancer/pro
DT-A
splice
polyA
T3 promoter
twin mcs
SauBAI
toxin mcs

Figure 19



3

Figure 20

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/01867

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5) : C12N 5/10,7/01,15/00,7/04,15/33,15/85
U.S. Cl : 435/172.3, 235,236,240.1,320

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	435/172.3,320,235,236,240.1; 935/32,57,60,70

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

CA & APS DATA BASES KEY WORDS: PACKAGING CELL, RETROVIRUS, HSV TK, TUMOR SPECIFIC ANTIGEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X	Transactions of the Association of American Physicians, Volume CI, 29 April- 02 May, 1988, MARKOWITZ ET AL, "Safe and efficient ecotropic and amphotropic packaging lines for use in gene transfer experiments", See page 213.	1-18
Y	US,A 4,687,737 (SHARP ET AL) 18 August 1987 See Columns 7-8.	1-18
Y	US,A 4,707,542 (FRIEDMAN) 17 November 1987 See Abstract.	1-4,6-9,
A	Science, Volume 236, Published 05 June 1987, (MANIATIS ET AL, "Regulation of Inducible and Tissue-Specific Gene Expression", Pages 1237-1245. See entire document.	1-18

* Special categories of cited documents: 15

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
03 July 1990	15 AUG 1990
International Searching Authority 1	**Signature of Authorized Officer** 20
ISA/US	for Sharon Nolan NGUYEN NGOC-HO INTERNATIONAL DIVISION

Form PCT/ISA/210 (second sheet) (May 1986)